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ON THE PRESENCE OF TYPHUS VIRUS IN WILD RATS IN NEW YORK CITY

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It is generally accepted at present that typhus fever can be transmitted in two ways; by the body louse from man to man, and by the rat flea from rat to man (cf. Mooser and associates,[1] Zinsser[2]).

Murine typhus virus has actually been recovered from rat fleas obtained at typhus foci in the United States by Dyer and his associates[3] and by Kemp,[4] in Manchuria by Kodama and his coworkers[5] and in Greece by Lépine and his associates.[6] The virus has also been recovered from the brains of wild rats in Mexico (Mooser, Castaneda and Zinsser[7]), on French battleships where typhus had occurred (Marcandier and Pirot,[8] des Essarts,[9] Marçon and Audoye[10]), in Greece (Lépine[11]), in Manchuria (Kodama and his associates),[5] in Syria (Lépine),[12] in the United States (Dyer and associates),[13] in Moscow

1. J. Exper. Med. **55:** 307, 1932; **59:** 137, 1934.
2. Am. J. Hyg. **20:** 513, 1934.
3. Pub. Health Rep. **46:** 334, 1931; J. A. M. A. **97:** 589, 1931; **99:** 795, 1932.
4. J. A. M. A., **97:** 775, 1931.
5. Kitasato Arch. Exper. Med. **9:** 84 and 91, 1932.
6. Compt. rend. Soc. de biol. **109:** 710, 1932; Bull. Acad. de Med., Paris **107:** 495, 1932; Bull. Soc. path. exot. **27:** 2, 1934.
7. J. A. M. A., **97:** 231, 1931.
8. Compt. rend. Acad. d. sc. **194:** 399, 1932; Bull. Soc. path. exot. **25:** 673, 1932; **26:** 349, 1933.
9. Bull. Soc. path. exot. **25:** 283, 1932.
10. Bull. Soc. path. exot. **25:** 390, 1932.
11. Compt. rend. Acad. d. sc. **194:** 401, 1932; Ann. Inst. Pasteur **51:** 290, 1933; Compt. rend. Soc. de biol. **117:** 848, 1934; Bull. Soc. path. exot. **27:** 2, 1934.
12. Compt. rend. Soc. de biol. **109:** 1072, 1932.
13. Pub. Health Rep. **47:** 2370, 1932. J. A. M. A. **99:** 795, 1932.

(Kritschewski and Rubenstein,[14] Kritschewski and Solowiow,[15] Epstein and Silvers),[16] in Morocco (Blanc, Noury, Baltazard and Fischer),[17] and in South Africa (Pijper and Dau).[18] In Alexandria, where Doorenbos[19] had isolated two strains of B. proteus X19 from wild rats, Panayotatou[20] found that 21 rats out of 50 showed positive Weil-Felix reactions, and expressed the opinion that rats thus constitute a typhus reservoir. Positive Weil-Felix reactions in wild rats in Lyons (Rochaix, Sédallian and Couture)[21] have also been reported.

As the result of their findings in Greece, Lépine and Bilfinger[6] are of the opinion that murine typhus may become epidemic among rats at times. In a series of experiments carried out during the months of June to December inclusive they found that among 230 rats positive Weil-Felix reactions varied from none in June and September to 1.6 per cent in October, 7–8 per cent in November and 23.8 per cent during the first part of December. During approximately the same period they examined the brains of 475 rats for the presence of typhus virus but succeeded in isolating it only twice, once in May and once in September. The authors were led to the conclusion that the Weil-Felix reaction indicated previous infection, the season with the high incidence having been preceded by a correspondingly high incidence of typhus infection in the same rat population.

Of considerable interest is the isolation of typhus virus from wild rats in localities where typhus does not occur clinically. Such reports have come from Paris (Brumpt),[22] from Bordeaux (LeChuiton and Moureau),[23] from Antwerp (Bruynoghe and Jadin),[24] and from Ham-

14. Centralbl. f. Bakt., O. **129:** 493, 1933.

15. Centralbl. f. Bakt., O. **131:** 232, 1934.

16. Gior. di batteriol. e immunol. **12:** 593, 1934 (Ab. Centralbl. f. Bakt., Ref. **115:** 431, 1934).

17. Compt. rend. Soc. de biol. **113:** 132, 1933.

18. South African M. J. **7:** 715, 1933 (cited in Centralbl. f. Bakt., O. **133:** 7, 1934).

19. Compt. rend. Soc. de biol. **102:** 290, 1929.

20. Compt. rend. Soc. de biol. **102:** 290, 1929; **111:** 430, 1932.

21. Compt. rend. Soc. de biol. **111:** 817, 1932.

22. Bull. Acad. de Med., Paris **107:** 356 and 416, 1932.

23. Compt. rend. Soc. de biol. **111:** 167, 1932.

24. Compt. rend. Soc. de biol. **113:** 399 and 1522, 1933; Arch. internat. de med. exper. **8:** 513, 1933.

burg (Suzuki).[25] In the light of such observations, the examination of rats in New York City, where typhus in the form of Brill's disease occurs occasionally, seemed desirable even though Zinsser's recent statistical investigation[2] led him to conclude that rats are not responsible for the occurrence of Brill's disease.

Fifty-one wild rats were obtained from the metropolitan area of New York City, for the most part from the neighborhood of wharves,

TABLE 1

Rat Number	Weight	Positive Weil-Felix Reaction	Guinea Pigs Injected with Rat Brain				
			Number	Amount Injected	Infection	Results of Tests for Immunity Using a Laboratory Strain of Typhus*: Maximum Temperature (C.)	Results of Tests for Immunity Using a Laboratory Strain of Typhus*: Scrotal Swelling
19	220 gm.	1–10 (faint trace)	5135	1/5 brain	No symptoms	39.4	Slight
31	240 gm.	1–40 (trace)	5153	1/5 brain	No symptoms	39.4	Slight
			5154	1/10 brain	No symptoms	40.	Marked
38	255 gm.	1–20 (trace)	5264	1/5 brain	No symptoms	39.5	Slight
			5265	1/10 brain	No symptoms	39.6	Slight
44	275 gm.	1–40 (trace)	5175	1/5 brain	No symptoms	39.4	None
			5174	1/10 brain	No symptoms	40.2	Moderate

* This strain of typhus when injected into normal guinea pigs produced, as a rule, febrile reactions with temperatures ranging between 40 and 40.5 C. accompanied with scrotal swelling of moderate to marked intensity.

during the months of September to December inclusive. All but 7 of these rats weighed over 100 grams, the large majority weighing over 200 grams. Of these 51 rats, 16 showed weakly positive Weil-Felix reactions, 9 with traces of agglutination in 1–10 dilution, 5 in 1–20 and 2 in 1–40. The brain of each of these 16 rats was emulsified and injected into two guinea pigs, using 1/5 and 1/10 of the brain respectively for intraperitoneal inoculation. In no case was typhus

25. Centralbl. f. Bakt., O. **131**: 236, 1934.

virus recovered and none of the 32 guinea pigs injected from these 16 rats developed a febrile reaction, but when subsequently tested with a laboratory strain of typhus, 5 showed what perhaps might be a certain degree of immunity (see table 1). The guinea pig injected with 1/5 brain from rat 44 (Weil-Felix 1–40) appeared upon reinjection to be immune (maximum temperature of 39.4 C., no scrotal swelling). However, another guinea pig which had received 1/10 of the same rat brain reacted with a typical infection when reinjected. Two guinea pigs which had received 1/5 brain respectively from rat 31 (Weil-Felix 1–40) and rat 19 (Weil-Felix 1–10) showed on reinjection maximum temperatures of 39.4 C. and only slight scrotal swellings. The pig which had received 1/10 brain from rat 31 developed typical symptoms. The guinea pigs injected with 1/5 and 1/10 brain respectively of rat 38 (Weil-Felix 1–20) showed upon reinjection maximum temperatures of 39.5 C. and 39.6 C. and only slight scrotal swellings.

In order to appraise the significance of the Weil-Felix reactions in this series, it should be mentioned that other observers who have examined wild rats found that the positive reactions were mostly of low titer (1–10 to 1–50), although reactions in higher dilutions are not infrequent (e.g. Kritschewski and Solowiow).[15] Positive Weil-Felix reactions in rats infected in the laboratory with typhus have been found in dilutions of 1–200 and 1–400 by Mooser[26] and in dilutions as high as 1–1,280 by Weigl.[27]

To summarize, it may be stated that although typhus virus was not actually recovered from the rats in this series, the finding of what appeared to be more or less marked immunizing effect following the injection of brain from rats which showed weak Weil-Felix reactions may possibly indicate previous typhus infection in such rats. The examination of a larger number of rats obtained from different localities during all seasons of the year seems indicated.

26. J. Infect. Dis. **44**: 186, 1929.
27. Bull. internat. Acad. polon d. sc. Lettres, Classe Med. July, 1930, 1.

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OBSERVATIONS ON THE GESTATION PERIOD OF THE RABBIT

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Although the rabbit has been used extensively in recent investigations on the physiology of reproduction, comparatively little information has appeared concerning the normal gestation period of this species of small laboratory animal. Hammond ('25) reported on the mean duration of pregnancy in 296 rabbits of the Belgian hare type. He found that 95 per cent of all observations fell between 30 and 34 days. Other authors refer to the gestation period of the rabbit variously as 30, 31, or 32 days. In connection with the studies on constitution which have been in progress in this laboratory during the past several years, and in order to arrive at a precise estimation of the duration of pregnancy in the rabbit, the pertinent data obtained from a large breeding colony have been subjected to a biometrical analysis. The results of this analysis form the basis of the present report.

Material and Methods

The results are based on an analysis of 1257 pregnancies in 716 does which terminated with viable young. The animals were all bred in a colony which is undergoing extensive study from the point of view of constitution (heredity). No attempt at selection of the material has been made, with the exceptions indicated below. The does and their mates are presumed to represent an unbiased sample of the general health and nutritional status which might be found in any large breeding colony. An occasional mother may have presented evidence of an intercurrent infection such as snuffles during or before the gestation period, but in general all were in good physical condition. None of the does were employed for experimental purposes other than those connected with breeding.

Animal material. Ten standard breeds, one intensively inbred line of albinos, and a group of hybrid matings are represented. Table 1

lists the number of does, the number of pregnancies, and the dates of the first and last mating in each of the twelve groups.

Each of the standard breeds has been inbred to a considerable extent in order to obtain a more homogeneous population than was represented by the original stock. In some cases close inbreeding has been maintained for several years. The breeds[1] range from the small fancy white Polish of cobby type which has a weight range of 1.3 to 1.6 kg., to the heavy, compact, varicolored Flemish, which

TABLE 1

1257 Pregnancies with Viable Issue in Twelve Groups of Rabbits

Group	Breed	Number of does	Number of pregnancies	First mating	Last mating
1	Albino	29	64	Jan. 9, 1929	Oct. 27, 1933
2	American Blue	13	17	Dec. 2, 1929	Nov. 3, 1932
3	Belgian	43	90	Nov. 29, 1929	Nov. 20, 1933
4	Blue Beveren	29	51	May 2, 1930	Nov. 21, 1933
5	Chinchilla	16	29	Nov. 18, 1929	Oct. 2, 1933
6	Dutch	36	64	Oct. 18, 1929	Dec. 15, 1933
7	English	34	64	Jan. 7, 1930	Dec. 29, 1933
8	Flemish	8	14	Nov. 18, 1929	Apr. 1, 1932
9	Havana	36	60	Oct. 9, 1929	Dec. 15, 1933
10	Himalayan	45	90	Mar. 27, 1929	Oct. 23, 1933
11	Polish	16	26	Feb. 13, 1930	Oct. 2, 1933
Pure breed total..........		305	569	Mar. 27, 1929	Dec. 29, 1933
12	Hybrid	411	688	Oct. 12, 1931	Nov. 17, 1933
Grand total..............		716	1257	Mar. 27, 1929	Dec. 29, 1933

averages from 5 to 7 kg. in weight. Between these extremes are the following breeds: The snaky Himalayan, a white animal with black points, weighing 1.8 to 2.7 kg.; the cobby Dutch, weighing 1.8 to 2.3 kg.; the compact, chocolate-colored Havana weighing 1.8 to 3.2 kg.; the standard Chinchilla, a compact gray animal, weighing 2.5 to 3.6 kg.; the slender English, weighing 2.7 to 3.6 kg.; the red-brown,

[1] For a description of American breeds and standards of perfection, the reader is referred to the Guide Book issued periodically by the American Rabbit and Cavy Breeders Association, Inc., Chicago, Ill.

racy Belgian hare, weighing 3.6 to 4.6 kg.; the Blue Beveren, weighing 3.2 to 3.6 kg.; and the American Blue, weighing 4 to 4.6 kg. The albino hybrid has been subject to extensive inbreeding in this laboratory over a period of years, and because of the resulting establishment of comparatively fixed characters, has been taken in this analysis to represent a pure breed or family of rabbit. This type of animal is sluggish, has a tendency to obesity, and weighs from 2.5 to 3.5 kg. The standard bred groups constitute matings between pure bred does and bucks of a given breed. The group included under hybrid matings represents crosses between the above standard breeds and a number of additional crosses with Lilac, Silver, Rex, Sable, Tan and Gouda breeds, and includes F_1, F_2, back-cross and miscellaneous hybrid matings.

Housing and feeding. The animals were confined in individual cages in well-ventilated rooms receiving daylight. Until the fall of 1930, they were fed a diet consisting of hay, oats and cabbage, with a restricted water ration. Since then the diet has comprised hay, oats, various commercial foods, and a free supply of water. Occasionally, the animals were hand fed with yeast, cod liver oil, or tomato juice alone or in combination to supply additional vitamins when needed or for test purposes.

Mating, births and estimation of duration of pregnancy. In all cases when a doe was to be mated, she was taken out of her cage and placed in the buck's cage. The pair were watched, and after copulation, the doe was returned to her own cage where she was allowed to remain until parturition. In many instances, however, she was placed in a large breeding cage when her condition required it. Matings were limited to the period between 9 A.M. and 5 P.M. and occurred on all days of the week except Sundays and holidays. It has not been the policy to note the exact time of copulation, but the date was carefully recorded.

Up to the time of this analysis, experience had shown that the gestation period ranged from 29 to 36 days, with an average of 31 days. Accordingly, the cage of each pregnant animal was examined early on the morning of the thirtieth day after mating, and each subsequent afternoon and morning in order to check up on the birth of a litter. When a litter had not been present at 5 P.M. of 1 day but was found on

the following early morning check, the birth was recorded as having taken place during the previous 16-hour (5 P.M. to 9 A.M.) interval. When the morning examination showed that a litter had not yet been born, while the late afternoon check showed the presence of a litter, the birth was recorded as of that day. In addition to the routine check-up the first thing in the morning and the last thing at night, expectant does were carefully watched during the day. The largest possible error was therefore, less than 16 hours, the interval between the evening and morning checks.

Two methods have been employed for estimating the gestation period of a particular doe. By the first method when a litter was born between 9 A.M. and 5 P.M., the gestation period was calculated from the day of mating to the day of birth in round numbers. If the litter was born between 5 P.M. and 9 A.M., the gestation period was estimated from the day of mating to the day before the morning when the litter was found, plus a correction of 8 hours or 0.3 day, the mid-point of the 16-hour interval between the 5 P.M. and 9 A.M. checks. The second method recorded all births between 5 P.M. of 1 day to 5 P.M. of the next day as of the latter day. In determining the class mean of this 1-day interval, 0.65 has been employed instead of 0.5 day. The reason for this procedure is evident from the fact that mating had occurred sometime between 9 A.M. and 5 P.M. of an antecedent day, and the class mean of 0.65 day includes a correction for this particular interval (9 A.M. to 5 P.M. = 8 hours; mid-point = 4 hours or 0.15 day). Repeated comparisons have shown that the two methods of calculation give similar results.

Beginning and ending with the dates for each of the twelve groups in table 1, the records for each group were systematically searched, and those of all pregnancies terminating with viable young were used for this analysis. Births which occurred during the period from Saturday noon to Monday morning and on holidays were excluded.

Statistical analysis. The usual statistical procedures have been employed, and the texts by Fisher ('30) and by Snedecor ('34) have been freely consulted. A mean difference was considered significant when it was at least two and one-half times its standard error ($t \geq 2.5$), that is, the probability of such a difference occurring by chance is 1 or less than 1 in 100 ($P \leq 0.01$). The method of analysis of vari-

ance in which the ratio F is used, is that described by Snedecor ('34), F being a symbol for the ratio of the larger to the smaller variance. The term 'variance' as used by Fisher indicates the square of the standard deviation, i.e., $V = \sigma^2$. The methods for calculating coefficients of correlation and transformed correlations, the method of 'z' and the χ^2 test of homogeneity are all described by Fisher ('30). In all these tests significance was attached to values of $P \leq 0.01$.

TABLE 2

The Gestation Period of the Rabbit. Distribution frequency of Pure Breed and Hybrid Values

Days	Albino	American blue	Belgian	Blue beveren	Chinchilla	Dutch	English	Flemish	Havana	Himalayan	Polish	Total pure breed	Hybrid	Grand total
29.0			2	1					1			4	3	7
29.3			2			2			1		3	8	18	26
30 0		1	3	2		3	1		4	4	5	23	32	55
30 3	1		6	6	3	13	11		13	16	10	79	168	247
31.0	1	6	26	12	7	17	17	3	15	30	6	140	119	259
31.3	1		19	13	11	18	15	6	16	22	2	123	204	327
32 0	15	3	11	4	6	6	9	1	3	7		65	47	112
32 3	12	3	12	6		5	5	1	2	7		53	70	123
33.0	14	2	5	4	1		2	2	3	3		36	15	51
33.3	6	2	2	2			3		1	1		17	6	23
34.0	7			1	1			1	1			11	3	14
34.3	3											3	3	6
35 0	1		1									2		2
35.3	1						1					2		2
36.0	2											2		2
36.3			1									1		1
Total.....	64	17	90	51	29	64	64	14	60	90	26	569	688	1257

RESULTS

The results are presented in a series of tables and figure 1. Table 2 gives the distribution frequencies of the gestation periods for each of the eleven pure breeds and their combined group of 569 values and the 688 values for hybrid matings. Table 3 summarizes these observations in terms of minimum, maximum, mean, standard deviation, standard error of the mean, and coefficient of variation. Table 4

TABLE 3

Summary of Observations on the Gestation Period of the Rabbit

Group	Breed	Number	Minimum	Maximum	Mean	σ	σ mean	Coefficient of variation
			days	*days*	*days*			*per cent*
1	Albino	64	30.3	36.0	32.89	1.07	0.13	3.25
2	American Blue	17	30.0	33.3	31.85	0.97	0.24	3.05
3	Belgian	90	29.0	36.3	31.46	1.09	0.12	3.46
4	Blue Beveren	51	29.0	34.0	31.46	1.01	0.14	3.21
5	Chinchilla	29	30.3	34.0	31.42	0.83	0.15	2.64
6	Dutch	64	29.3	32.3	31.04	0.70	0.09	2.26
7	English	64	30.0	35.3	31.41	0.85	0.11	2.71
8	Flemish	14	31.0	34.0	31.79	0.92	0.25	2.89
9	Havana	60	29.0	34.0	31.08	0.92	0.12	2.96
10	Himalayan	90	30.0	33.3	31.18	0.79	0.08	2.53
11	Polish	26	29.3	31.3	30.37	0.57	0.11	1.88
Pure breed total..........		569	29.0	36.3	31.45	1.08	0.05	3.43
12	Hybrid	688	29.0	34.3	31.11	0.86	0.03	2.77

TABLE 4

Summary of Observations on the Duration of 1257 Pregnancies Resulting from Pure Breed and Hybrid Matings

Mean of class	Number of observations	Per cent of total	Constants	
days				
28.65	7	0.6	Minimum,	29.0
29.65	81	6.4	Maximum,	36.3
30.65	506	40.3	Mean,	31.34
31.65	439	34.9	Median,	31.23
32.65	174	13.8	Modal class,	30–31
33.65	37	2.9	Standard deviation,	1.13
34.65	8	0.6	Standard error of standard deviation,	0.023
35.65	4	0.3	Standard error of mean,	0.032
36.65	1	0.1	Coefficient of variation,	3.61%
			γ_1, $+ 1.088 \pm 0.0047$	
			γ_2, $+ 2.011 \pm 0.0191$	

gives an analysis of the combined pure breed and hybrid group of 1257 observations in these terms, and presents additional values for the median, modal class, standard error of the standard deviation, and for

γ_1 and γ_2. For estimating the duration of pregnancy the first method described in the section on Material and Methods has been followed throughout with the exception of the values included in table 4 and figure 1, which have been derived by an application of the second method. The latter procedure was followed because equal class intervals simplified the construction of a frequency polygon.

Tables 2 and 3 and figure 1 show that wide variations exist between mean values for different breeds. It is seen that the Polish breed had the shortest mean gestation period of 30.37 days and the albino the longest, 32.89 days. The values for the Belgian group showed the

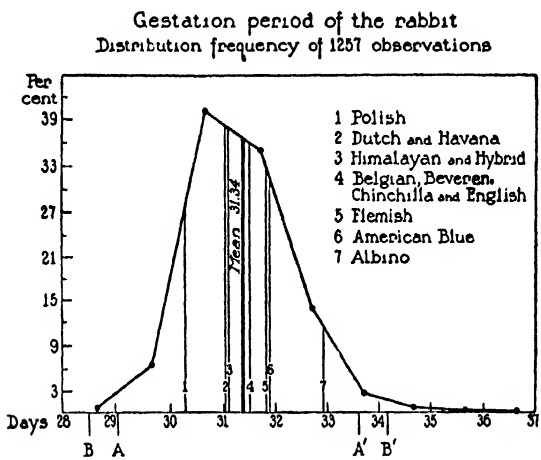

FIG. 1

greatest spread as indicated by the coefficient of variation of 3.46 per cent, while the Polish breed had the most compactly grouped values, giving a coefficient of variation of 1.88 per cent. There were seven gestation periods lasting 35 days or more with the birth of living young. Four of these were in the albino group, two in the Belgian, and one in the English. The significance of the different breed mean values will be discussed later.

Figure 1 presents a frequency polygon which summarizes the 1257 observations, and indicates the position of the several pure breed mean and hybrid mean values. The observations included between the letters A and A′ are separated from the grand mean of 31.34 days by a

distance equal to twice the standard deviation. The gestation period of the normal rabbit would not be expected to fall outside these two values of 29.1 and 33.6 days more often than once in twenty-two times. The letters B and B′ are separated from the grand mean by a distance equal to two and one-half times the standard deviation and values for the normal rabbit would not be expected to fall outside these extremes of 28.5 and 34.2 days more often than once in 100 times. The value γ_1 indicates that the frequency distribution curve illustrated is significantly skewed to the right, and the value γ_2 indicates that this distribution does not follow the normal curve of error, being heaped too high at the top and cut too thin at the shoulders.

The present study indicates that, provided the selection constitutes a fair sample, and the real distribution is normal, gestation periods ranging from 29.1 to 33.6 days are normal, while gestation periods shorter than 28.5 days and longer than 34.2 days are abnormal.

DISCUSSION

In evaluating the results obtained, certain variables which might influence the duration of pregnancy were more precisely analyzed. These have been classified as extrinsic and intrinsic factors. In the first group will be considered the method of computation of the gestation period and the influence of diet and season. Among the intrinsic factors are the age of the doe at the time of mating, the breed and relative weight of the doe, and the influence of litter size, repeated pregnancies, hybridization, and of the buck on the gestation period of his mate. The influence of litter weight is beyond the scope of the present report and will be presented in detail at a later date.

A. Extrinsic Factors

1. Methods for estimating gestation period. It has been stated in a previous paragraph that the two methods of computing the duration of pregnancy for a particular doe as described in the section on Material and Methods gave similar results. This statement is substantiated by the following analysis. The mean duration of pregnancy for the 1257 observations calculated by the first method was 31.26 ± 0.028 days, while an analysis of the same observations in which duration was computed by the second method gave a mean value of 31.34

± 0.032 days. The difference of 0.08 ± 0.042 is less than twice its standard error and is statistically insignificant. The two methods are, therefore, statistically comparable.

2. Diet. The influence of dietary factors could not be determined since during the entire period under consideration, an adequate well-balanced ration was maintained. There is some evidence, however, that faulty diets, or constitutional inability to metabolize adequate diets may be responsible for alterations in the duration of pregnancy.

TABLE 5

Gestation Period and Season of the Year

Breed	Jan.-Feb.		March-April		May-June		Sept.-Oct.		Nov.-Dec.	
	Number	Mean	Number	Mean	Number	Mean	Number	Mean	Number	Mean
		days		*days*		*days*		*days*		*days*
Albino	20	32.74	12	33.40	7	32.47	7	32.80	18	32.81
American Blue	3	31.33	3	32.43			4	32.40	7	31.51
Belgian	17	30.99	20	32 02	9	32.27	23	31 28	21	31 20
Blue Beveren	12	31.42	12	31.22	2	32.00	13	31.73	12	31.35
Chinchilla	9	31.36	6	31.37	2	32.65	5	31.52	7	31.27
Dutch	15	30.91	17	31.17	5	30.92	11	31.27	16	30.90
English	16	30.97	18	31.49	3	32.33	18	31.42	9	31.64
Flemish	4	31.47	3	31.33			2	31.30	5	32.52
Havana	15	30.92	11	31.28	1	31.00	17	30.91	16	31.33
Himalayan	22	31.10	23	31.18	6	30.83	21	31.25	18	31.21
Polish	5	30.66	8	30.23	4	30.15	5	30.58	4	30.22
Total observations	138	31 31	133	31 54	39	31.67	126	31.47	133	31.48
Total breeds	11	31.26	11	31.47	9	31 61	11	31.50	11	31.45

The evidence on this point is derived from experience and is too sketchy to be submitted to statistical analysis.

3. Season (table 5). An analysis of the observations on the standard breeds indicated that seasonal factors at the time of mating did not exert a significant influence on the duration of pregnancy. The mean value of 31.51 ± 0.07 days calculated for all pure breed matings in the interval between March and October was not significantly different from the mean value of 31.39 ± 0.06 for the period November to February (difference = 0.12 ± 0.09, t = 1.3). An alternative

analysis was made by the method of variance. Breed mean values for the five 2-month intervals from January to June were calculated for each breed (table 5). The variance of the breed mean values within 2-month classes was greater than the variance of the meanings of the breed mean values between 2-month classes (V within 2-month classes = 0.54; V between means of 2-month classes = 0.45, F = 1.2). Similarly, a calculation on the basis of all individual values regardless of breed, gave the following result: V within 2-month classes = 1.12; V between means of 2-month classes = 1.22, F = 1.09. Although here the variance between classes is slightly greater than the variance

TABLE 6

Age of Standard Bred Does at the Time of Mating

Breed	Number	Maximum	Minimum	Mean	σ	σ Mean
		months	*months*	*months*		
Albino	56	28.0	4.7	12.7	5.57	0.75
American Blue	15	11 8	6.1	10.0	1.72	0.46
Belgian	81	36.3	4.7	13.5	6.93	0.78
Blue Beveren	51	28.3	4.3	12.8	6.25	0.88
Chinchilla	25	40.1	4.6	14.8	9.13	1.86
Dutch	57	30.2	3.9	11.8	6.17	0.83
English	64	45.4	4 3	14.3	8.76	1.10
Flemish	14	20.5	7.3	10 8	3.14	0.76
Havana	57	34.0	5.6	13.7	6.75	0.90
Himalayan	76	26 4	4.4	12.7	5.73	0.67
Polish	26	23.3	4.9	11.7	4.97	0.99
Total	522	45 4	3.9	13.0	6.69	0.29

within classes, the difference (z = 0.0855) is not significant. The largest difference between any pair of means for 2-month classes was that between the May–June class with a mean of 31.67 days and the January–February class with a mean of 31.31 days. The difference of + 0.36 ± 0.19 days is not significant since it is less than twice its standard error. The indication is that with respect to season the pure breed values are homogeneous.

B. Intrinsic Factors

1. Age of doe at time of mating. Table 6 presents the values for the age of the standard bred does at the time of mating. It will be noted

that 522 or 91.7 per cent of the total of 569 pregnancies are represented. This discrepancy is due to the fact that the age of some of the original stock animals which had been purchased from outside sources was not definitely known. However, the observations comprise an adequate sample of the total pure breed pregnancies. The breed mean age of the doe at the time of mating varied from 10.0 ± 0.46 months for the American Blue breed to 14.8 ± 1.86 months for the Chinchilla, with a grand mean for all observations of 13.0 ± 0.29 months. The youngest doe was a Dutch mated at 3.9 months and the oldest an English mated at 45.4 months. The method of analysis of variance gave significant evidence that the mean age of the doe was the same for the different breeds, and that the whole set of 522 values represented a homogeneous population (V between means of breeds = 5.91; V within breeds = 45.64, F = 7.72, P = 0.01−). Moreover, correlating the breed mean gestation period with the mean age of the doe at the time of mating gave an insignificant negative value of r_{xy} = −0.156. It was concluded that within the limits specified the doe's age had no influence on the duration of pregnancy.

2. *Breed.* Table 2 and figure 1 show the variation in the mean values for different breeds. Not only were different gestation periods found for different breeds, but breeds closely related by origin or type, such as the Havana and Dutch, and the Belgian and English, had identical mean gestation periods, while comparatively unrelated breeds had widely different periods. Moreover, the variance between the means of breeds was significantly greater than the variance within breeds (V between means of breeds = 19.09; V within breeds = 0.86, F = 22.22, P = 0.01−). In order to make the test more exacting, the albino group which had the longest mean gestation period of all the breeds was excluded from the calculation. On this basis, the variance between breeds, although reduced, was still significantly greater than the variance within breeds (V between means of breeds = 4.66, V within breeds = 0.76, F = 6.16, P = 0.01 −). The demonstration of heterogeneity between breeds is taken to indicate that with respect to the fact or under consideration, each breed represented a homogeneous population. In a previous publication (Rosahn, et al., '34) it was concluded that the differences between the gestation periods of different breeds were largely dependent on hereditary factors.

3. Weight of the doe. There is evidence that body weight of the doe affects the duration of pregnancy. In general, the lighter breeds such as the Polish and Himalayan had shorter mean gestation periods than the heavier breeds such as the American Blue and Flemish. This relationship was not constant since the gestation period of the comparatively small English was the same as that for the heavier Beveren. However, when the breeds were divided into two classes according to weight, the gestation period of the heavier group of Beveren, Belgian, American Blue, and Flemish was significantly longer than that for the lighter group of Himalayan, Dutch, Polish, and Havana (mean of heavy group = 31.52 ± 0.079; mean of light group = 31.03 ± 0.055; difference = $+ 0.49 \pm 0.097$; $t = 5.0$, $P = 0.01 -$). This calculation excluded the comparatively heavy albino group with its long gestation period in order to eliminate bias. The mean gestation period of the intermediate group of English and Chinchilla rabbits was longer than that for the light group and shorter than the mean for the heavy group (mean of intermediate group = 31.42 ± 0.080). Since weight is one of the standards by which breed is determined, it is not surprising to find that breed weight is related to gestation period in view of the above demonstration that the gestation periods of different breeds of rabbits differ significantly.

4. Number in litter. It is seen from table 7 that the Polish group with the shortest mean gestation period had the smallest mean value for litter size. Excluding the albino group, at the other extreme was the Flemish breed with the longest mean gestation period and the largest mean number of animals per litter. This relationship is indicated by a probably significant correlation coefficient of $+ 0.708$ between the values indicated in table 7 for breed mean gestation period and breed mean litter size. The albino mean values fall definitely out of line with the other observations and were not included in the calculation of the above coefficient. Some extraneous factor was clearly responsible for this irregularity, which we are inclined to believe was due to the long period of intensive inbreeding of this group with the concentration of factors which tended to affect reproduction adversely.

The evidence also indicates that for any given breed the gestation period is longer for small than for large litters (table 7). With the

exception of the English group, all the coefficients of correlation between individual values for gestation period and litter size within a breed are negative in sign, and combining the eleven breed coefficients by the method of transformed correlations gives a significant negative value of − 0.3004 (P = 0.01−). Thus, between breeds prolonged gestation periods were associated with large litters and short gestation periods with small litters. In marked contrast to this, within any given breed, prolonged gestation periods were associated with small litters and short gestation periods with large litters.

TABLE 7

Gestation Period and Litter Size

Breed	Mean gestation period	Mean litter size	Correlation coefficient between individual values for gestation period and litter size
	days		
Albino	32.88	4.33	−0.633
American Blue	31.85	5.76	−0.483
Belgian	31.46	5.10	−0.308
Blue Beveren	31.46	7.14	−0.548
Chinchilla	31 42	4.97	−0.299
Dutch	31.04	5.48	−0.232
English	31 41	6.05	+0.267
Flemish	31 79	7.14	−0.735
Havana	31 08	5.38	−0.412
Himalayan	31.17	4.72	−0 172
Polish	30.37	3.92	−0.048

5. *Pregnancy number.* A further analysis was concerned with the influence of repeated pregnancies on the gestation period. A tabulation was made of each pregnancy of the 305 pure bred does, listing the duration and the number, whether first, second, third, etc. From this tabulation were excluded those items representing the first and only pregnancy of the doe, and also those pregnancies which terminated in abortion, in non-viable young, or over a week end. The gestation periods of the fifth and succeeding pregnancies were grouped in one class. It will be seen from the resulting tabulation (table 8) that the mean values decrease from a high level of 31.62 days for the first pregnancy, to 31.36 days for the third, and rise to a value of 31.58

days for the fifth and later pregnancy group. A statistical analysis of these figures without regard to breed does not indicate any significance to these differences. By the method of analysis of variance, the variance within the pregnancy number classes is greater than the variance between the means of pregnancy number classes (V within pregnancy number classes = 1.212; V between means of pregnancy number classes = 1.000). The variance within classes was actually the greater, with an observed difference (z) of 0.1906, which, however,

TABLE 8

Gestation Period and Pregnancy Number

Breed	First		Second		Third		Fourth		Fifth and etc.	
	Number	Mean	Number	Mean	Number	Mean	Number	Mean	Number	Mean
		days		*days*		*days*		*days*		*days*
Albino	17	32.89	19	33.11	9	32.17	6	33.00	3	33.00
American Blue	3	31.67	6	32 05	1[1]	31.00				
Belgian	17	31.46	25	31.44	16	31.51	10	31.02	10	31.91
Blue Beveren	9	31.58	14	31.44	8	30.98	5	32.12	3	32.30
Chinchilla	6	31 83	4	31.33	3	32.10	4	31.43	3	30.97
Dutch	20	31.12	21	31.12	9	30 90	5	31.12	1[1]	31.30
English	11	31.53	15	31.47	9	31 61	3	31 43	8	31.31
Flemish	3	32 43	4	31.83	1[1]	32 30	2	31 65		
Havana	10	31.19	13	31.01	5	31.18	3	31.77	9	31.53
Himalayan	22	31.44	22	31.22	15	31.21	8	30.78	4	30.98
Polish	4	30.75	6	30.53	4	30.23	4	30.15	1[1]	29.30
Total observations	122	31.62	149	31.54	80	31.36	50	31.39	42	31.58
Total breeds	11	31.63	11	31.50	9	31.32	10	31.45	7	31.71

[1] Omitted from calculation of mean of breeds.

is an insignificant value. The whole set of 443 values appears to be homogeneous, and statistically, no significant influence on the gestation period could be ascribed to repeated pregnancies.

6. Hybridization. The first method of estimating the gestation period gave a mean value for the 569 pure breed pregnancies of 31.45 $\pm$ 0.05 days, while a similarly obtained value for the 688 hybrids was 31.11 $\pm$ 0.03 days. The difference of 0.34 $\pm$ 0.056 days is a significant value, since it is six times the standard error of the difference. Even

when the albino group with its long gestation period was eliminated from the pure breed mean value, it was still significantly larger than the hybrid mean value (Pure breed: $n = 505$, $\text{Mean}_n = 31.27 \pm 0.04$; Hybrid: $n' = 688$, $\text{Mean}_n' = 31.11 \pm 0.03$, Difference $= 0.16 \pm 0.053$, $t = 3.0$, $P = 0.01-$).

The skew to the right is greater for the pure breed values than for the hybrids (table 2). Seventy-four of 569 pure breed values or 13.0 per cent were 33.0 days or longer, while only 27 of 688 hybrid values or 3.9 per cent were 33.0 days or longer. The difference is significant ($\chi^2 = 33.92$, $P = 0.01-$). Excluding the albinos from the pure breed calculation of 505 values, 40 or 7.9 per cent were 33.0 days or more, which is still significantly larger than the hybrid value of 3.0 per cent ($\chi^2 = 8.71$, $P = 0.01-$). The greater spread of the pure breed values is indicated by its larger standard deviation of 1.08 ± 0.032, which is significantly larger than the standard deviation of the hybrid values, 0.86 ± 0.023 (difference $= 0.22 \pm 0.039$, $t = 5.6$, $P = 0.01-$). Hybridization thus seems to eliminate extremes, and groups the values more compactly about the mean.

Although this is true grossly, a more careful scrutiny of the values indicates that greater fluctuations occur in the hybrid observations. Thus in table 2 the frequencies of the pure breed values describe a uniform upward trend to the highest frequency of 140 in the 31.0 day grouping followed by a uniform downward trend. The trend of the hybrid frequencies, on the other hand, is jagged with a frequency of 168 for the 30.0-day interval followed by a fall to 119 for the 30.3-day interval, then a rise again to 204 for the 31.0-day interval, then a fall to 47, and a rise again to 70. In summary, this analysis indicates that hybrid values were more concentrated but showed many more smaller fluctuations than did pure breed observations. From a genetic point of view these findings are of great interest since they show that although hybridization eliminates extremes, it tends to exaggerate finer differences.

7. *The male parent.* An attempt has been made to analyze the influence of the buck on the gestation period of the doe with which he has mated. For this purpose, all the matings between pure Himalayan does and pure Polish bucks were tabulated. Twenty such matings were recorded, with a mean gestation period of 30.62 ± 0.20 days.

This value is smaller than the pure Himalayan mean value of 31.18 $\pm$ 0.08, from which it significantly differs, and larger than the pure breed Polish mean value of 30.37 $\pm$ 0.11 days. These observations are in accord with those of Nathusias, who found in sheep that the early maturing Southdown had an average gestation period of 144 days while the larger, late maturing Merino had one of 150 days. A lamb got by a Southdown ram from a Merino ewe was carried a shorter time than one by a Merino ram. It is apparent that at least in some instances the buck has an influence on the gestation period of his mate. Whether this influence is operative through the differing weight or number of the hybrid offspring cannot at present be shown. The evidence presented involved the mating of a doe, the Himalayan with lighter bucks, the Polish, resulting in a gestation period which was shorter than the mean value for the doe's breed and longer than that for the buck's. Unfortunately, we did not have a sufficient number of matings between a light breed doe and a heavy breed buck to demonstrate whether the resulting gestation period would be longer than the mean value for the doe's breed and shorter than that for the male parent's.

SUMMARY

1. An analysis has been made of the duration of 569 pregnancies in 305 pure bred rabbits representing eleven breeds and 688 pregnancies in 411 hybrid rabbits. It was estimated that gestation periods between the extremes of 29.1 days and 33.6 days should be considered normal, while gestation periods shorter than 28.5 days or longer than 34.2 days should be considered abnormal.

2. Diet appeared to have an influence on the duration of pregnancy although the mechanism and direction of this influence could not be determined from the reported observations.

3. Seasonal factors at the time of mating did not exert a significant influence on the duration of pregnancy.

4. The age of the doe at the time of mating did not significantly affect the period of gestation.

5. Breed was found to exert a significant influence on the period of gestation. Comparatively unrelated breeds had widely different mean gestation periods, while breeds closely related by origin or type had identical mean gestation periods.

6. The body weight of the doe significantly influenced the duration of pregnancy. In general, the lighter breeds had shorter mean gestation periods than the heavier breeds.

7. Within any given breed, prolonged gestation periods were associated with small litters and short gestation periods with large litters. However, there was a positive correlation between breed mean gestation period and breed mean number in the litter.

8. No statistically significant influence on the duration of pregnancy could be ascribed to repeated pregnancies.

9. The pure breed mean gestation period was significantly longer than the mean gestation period for hybrids. Moreover, the hybrid values were more concentrated but showed many more smaller fluctuations than did the pure breed observations.

10. It was found that at least in some instances the male parent significantly influences the gestation period of his mate.

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CELLULAR REACTIONS TO WAX-LIKE MATERIALS FROM ACID-FAST BACTERIA

THE UNSAPONIFIABLE FRACTION FROM THE TUBERCLE BACILLUS, STRAIN H-37

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PLATES 32 AND 33

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The Mycobacteria are discriminated as a group by their capacity for synthesizing large amounts and varied types of lipoids in the form of fatty acids, phosphatides, and wax-like substances. Since no sterols have been found in them (1), the materials which have the properties of waxes are not waxes in the chemical sense. Rather they have been found to be composed of higher solid alcohols (2), and hydroxy acids of high molecular weight combined with polysaccharides, or, in the case of the corresponding substances from the *Bacillus leprae*, glycerides (3, 4). Like waxes, however, these materials are not only completely insoluble in water, but they cannot even be wet with water, properties which offer difficulties to the study of their effects on cells. Nevertheless, it can be shown that they are powerful stimulants for the new growth of cells. These wax-like materials have the property of acid-fastness and contribute it to the bacilli. For convenience we shall speak of these compounds as waxes.

As is well known, the separation of mixtures of lipoids is only to be made by the appropriate use of solvents. In general, Anderson has found that with the first use of each solvent, some of all the different types of lipoids come out. When it is recalled that almost all of the early biological tests of tuberculo-lipoids were made upon original alcohol-soluble, or alcohol-ether-soluble, or chloroform-soluble extracts, it is easy to understand why the effects so induced were complex and brought out no single cell reactions. Dr. Anderson has separated these lipoids from each other and given us three types of material.

The first, the so called acetone-soluble material, is a mixture of many fatty acids and is so irritating that it stimulates every type of connective tissue cell (5). The other two preparations, on the other hand, the phosphatides and the waxes, give practically a single cell reaction. Both of these materials stimulate monocytes, but their effect on the monocyte is different. With the phosphatide, monocytes become epithelioid cells and form tubercles in all their essential characteristics; while on the other hand, the waxes bring about a fusion of monocytes into foreign body giant cells. This paper is concerned primarily with the reactions to the unsaponifiable material or wax which will be considered in comparison with those to the phosphatide.

Materials and Methods[1]

The Unsaponifiable Wax from Human Tubercle Bacilli, H-37.—This wax was obtained by Anderson (2) from the bacterial residue remaining after the extraction with alcohol-ether. The residue was treated with chloroform and the extract, after evaporation of the chloroform, gave a large amount of crude wax. This was then dissolved in ether, to which either acetone or methyl alcohol was added. A precipitate formed which was then separated into a saponifiable and an unsaponifiable portion by boiling in alcoholic potassium hydroxide. It is the unsaponifiable portion which is acid-fast and which contributes the wax-like characteristics to the chloroform extract. Some of this unsaponifiable material was also obtained from the original ether-alcohol extract both in the process of purification of the phosphatide and from the acetone-soluble material. After purification it is a white, amorphous powder, soluble in ether or chloroform, insoluble in water, and extremely stable. It is an hydroxy acid with the formula $C_{94}H_{188}O_4$ (1).

In all of the earlier work in this laboratory with the waxes, including the crude wax as well as the more purified, the materials were introduced in solution in mineral oil (6). Since mineral oil itself, the most inert oil yet found, causes considerable reaction by inducing a new growth of fibroblasts, by inducing adhesions, and by being phagocytized by clasmatocytes (macrophages), we have now devised other methods of introducing the waxes. The intraperitoneal route has been used for the most part, but the material has also been introduced subcutaneously, intradermally, and intravenously.

The wax has been injected intraperitoneally in three ways: first, as a dry

[1] All of the materials we have used in these experiments have been obtained from the analyses of Dr. R. J. Anderson and his coworkers, Sterling Chemistry Laboratory, Yale University, and the work is a part of a plan for cooperative research sponsored by the Research Committee of the National Tuberculosis Association, of which Dr. William Charles White is Chairman.

powder through a cannula and either pushed in by trocar or blown in with air; second, as a dry powder through an incision under anesthesia; and third, in colloidal suspension. The first method proved the least instructive, because the wax-like materials frequently became packed into a single bolus within the needle. The second method is the most satisfactory for all of the different materials. It is possible, if aseptic precautions are used, to open the peritoneal cavity several times and dust the powdered waxes onto the omentum. The crude waxes, which were in large lumps, could be ground into fine powder if they were first chilled with dry ice until brittle. The wax often became charged electrically when scraped from the watch glass into the peritoneal incision. This, however, did not alter the cellular reactions.

The colloidal suspensions proved instructive on account of the opportunity they afforded to introduce these inert materials in fine particles. They were made by one of us as follows: A given weight of the unsaponifiable wax from H-37 was dissolved in chloroform and then an equal amount of hot alcohol was added. The material remained in solution. When an equal amount of distilled water was added drop by drop, the wax came out in a precipitate of particles so fine as to make a milky suspension. The flask was then placed in a water bath and kept at 100°C. until all of the chloroform and alcohol had been driven off. The water was then concentrated until 1 cc. contained 5 mg. of the wax. This suspension proved to be stable with no aggregation of the precipitate into clumps on standing.

RESULTS

Reactions to Unsaponifiable Wax from H-37 Given Intraperitoneally.—The unsaponifiable material from tubercle bacilli, H-37, has been given intraperitoneally to seventeen rabbits in amounts shown in Table I. All of these animals showed also the reaction to the subcutaneous injection, since small amounts always lodged there in introducing the wax. In the table the animals are arranged according to the time which elapsed from the first injection to the date of killing the animal.

In the peritoneum the reaction to the wax is under the mesothelium of the parietal peritoneum, in the serosal coat of the cecum, in the capsule of liver and spleen, and in the omentum. The descriptions in the table are mainly of the omentum as representative of the entire reaction, because of the advantages in studying this structure. It can be studied as a film, while the cells are living, showing the supravital reaction to neutral red and Janus green. Then the same preparation can be fixed and stained in some manner, such as with the Ziehl-

TABLE I

Protocols of Rabbits Which Received Unsaponifiable Wax from Tubercle Bacillus, H-37, Intraperitoneally

Animal No.	No. and amount of injections	Time	Method of preparing material	Peritoneal exudate: Percentage of PMN	Lymph.	Mono.	Tissues
		days					
R 3351*	1 24 mg.	1 K†	Colloidal suspension	41.5	2.6	55.8	Acid-fast material on milk spots of omentum and none in interspaces. 10% of monocytes in peritoneal exudate contain acid-fast material
R 2760	1 20 mg.	4 K	Dry powder through cannula	17.1	8.8	74.1	Milk spots of omentum show an increase in monocytes surrounding wax, but they have not started to fuse into foreign body giant cells. Infiltration with eosinophilic leucocytes
R 2793	1 20 mg.	4 K	Dry powder through incision under anesthesia	2.55	34.7	62.75	Wax became highly charged electrically and lodged in fat around the kidney, where foreign body giant cells developed. No infiltration with eosinophilic leucocytes
R 2840	2 20 mg.	4 2 D‡	" "	—	—	—	Died of pneumonia. Foreign body giant cells in omentum
R 2884	1 20 mg.	6 K	" "	—	—	—	Wax in small particles on milk spots, surrounded by newly formed monocytes which are starting to fuse into foreign body giant cells. Coccidiosis present
R 3301	2 20 mg.	4 3 K	Colloidal suspension	3.3	19.8	76.85	Milk spots show dense masses of monocytes around wax; some have fused into foreign body giant cells. Reaction to acid-fast stain brilliant; it is in part free on the milk spots and in part within the monocytes and giant cells. Infiltration with eosinophilic leucocytes. Small foci of monocytes in septa between air sacs in lungs

R 3322	1 20 mg.	8 K	" "	0	3.0	96.5	Milk spots increased in size by new monocytes around particles of wax; some fusion into foreign body giant cells. Only a little acid-fast material within the cells but much on the milk spots. Infiltration with eosinophilic leucocytes. Foreign body giant cells in retrosternal lymph nodes. Many young monocytes in peritoneal fluid
R 2802	2 20 mg.	4 4 K	First through cannula; second through incision under anesthesia	—	—	—	First injection lodged in a bolus. Second caused increase in monocytes around particles of wax on the milk spots. Some fusion into foreign body giant cells. In second incision there was a marked increase in monocytes and infiltration with eosinophilic leucocytes. Increase in eosinophilic myelocytes in the bone marrow
R 2841	2 20 mg.	4 4 D	Dry powder through incision under anesthesia	—	—	—	Died of pneumonia. Omentum, adherent to peritoneal wall at site of operation, contains foreign body giant cells and has infiltration with eosinophilic leucocytes
R 2814	2 20 mg.	3 14 K	" "	0	33.7	66.3	Marked increase in size of milk spots, each of which has from 2 to 10 foreign body giant cells. In sections some of the giant cells have a hollow center, from which the wax has been dissolved out, but show no vacuoles in the fused cytoplasm. In others wax had been phagocytized. Infiltration with eosinophilic leucocytes. Nodule in skin of first incision has foreign body giant cells with partially fused cytoplasm. In second incision masses of monocytes but relatively little fusion into giant cells. Many eosinophilic leucocytes in incisions. Retrosternal lymph nodes have many complex giant cells

PMN = polymorphonuclear neutrophilic leucocytes. Lymph. = lymphocytes. Mono. = monocytes.

* These are serial experiment numbers covering the entire work of the department.

† K = Killed.

‡ D = Died.

TABLE I—*Concluded*

Animal No.	No. and amount of injections	Time	Method of preparing material	Peritoneal exudate: Percentage of PMN	Peritoneal exudate: Percentage of Lymph.	Peritoneal exudate: Percentage of Mono.	Tissues
		days					
R 2806	2 20 mg.	4 23 K	Dry powder through incision under anesthesia	0	46.5	53.0	Milk spots increased in size with monocytes and foreign body giant cells. 8 to 10 giant cells in each milk spot. Moderate infiltration with eosinophilic leucocytes. Sections show tubercles of giant cells
R 3323	1 20 mg.	30 K	Colloidal suspension	0	5.1	94 9	Milk spots of omentum not increased in size but show foreign body giant cells which, as well as the monocytes, show small acid-fast granules surrounded by a pink zone in Ziehl-Neelsen stain. Most of the reaction was in the retrosternal lymph nodes, where the sinuses were filled with small giant cells having a uniformly vacuolated cytoplasm
R 3325	1 20 mg.	32 K	" "	0.5	17.35	82.15	Milk spots of omentum massive with foreign body giant cells. Only the giant cells show any acid-fast material and this consists of tiny granules stained red and surrounded by a pink zone in Ziehl-Neelsen
R 3326	1 20 mg.	46 K	" "	1.03	2.06	96.90	Milk spots of omentum dense with foreign body giant cells, some of which have irregular masses and finely divided particles that stain faintly acid-fast
R 2839	1 20 mg.	55 K	Dry powder through incision under anesthesia	0	39.2	60.8	Milk spots of omentum massive with tubercles of foreign body giant cells; tissues infiltrated with lymphocytes and with some eosinophilic leucocytes. Cytoplasm of the giant cells is vacuolated. There is some calcification but nothing that simulates caseation

R 2845	2 20 mg.	4 133 K	"	"	0	8.0	91.0	Milk spots of omentum massive with foreign body giant cells. Similar masses in the body wall. All of the tissues infiltrated with some eosinophilic leucocytes; some calcification. Nodules of monocytes and eosinophilic leucocytes in the lung
R 2844	2 20 mg.	6 222 K	"	"	0	56.65	43.35	Milk spots of omentum massive with tubercles of foreign body giant cells. Some masses of giant cells raised on pedicles. Supravital reaction to neutral red showed that the wax had become much more finely divided in the cells than in R 2845. Some plasma cells and an occasional eosinophilic leucocyte

Neelsen technique. Other preparations of the omentum can be fixed for sections. Besides occurring in these locations, some of the wax, occasionally much of it, floods through the lymphatics of the diaphragm into the retrosternal lymph nodes. In most instances, small particles also reach the lung and are there represented by small foci of monocytes usually infiltrated with eosinophilic leucocytes.

The immediate reaction to the material injected in the form of the colloidal suspension is instructive. The material is identified by its property of acid-fastness.

The colloidal suspension itself shows only a diffuse, pink reaction to the fuchsin of the Ziehl-Neelsen technique, for the wax has to be in aggregates of a given size before the typical red color of the reaction can be obtained. Aggregates of the material are formed when the colloidal suspension is introduced intraperitoneally and the acid-fast reaction is shown in Fig. 1, from the omentum of Rabbit R 3351 which was killed 24 hours after one injection of 24 mg. This film was fixed in the vapor of formalin and stained with the Ziehl-Neelsen method. The wax, stained red with the fuchsin, appears black in the photograph and is almost limited to the milk spots.

It was clear on studying the fresh films of the omentum of this animal, R 3351, that the wax was merely adherent to the cells of the milk spots and had not been phagocytized by them. The cells of the peritoneal exudate were stained for acid-fast material and only 10 per cent of the monocytes contained any of this material and they only in small amounts.

In the fifth column of Table I is shown the differential counts of the cells of the peritoneal exudate expressed in percentages of the three strains of cells which are significant. In some of the counts there was an occasional basophilic leucocyte, never more than 2 per cent; but in none was there even a single eosinophilic leucocyte; in some counts there were a few clasmatocytes with phagocytized leucocytes and occasionally desquamated serosal cells. These cell types may be considered as accidental and hence have been omitted in the table. Only the first two animals listed in Table I showed any rise in neutrophilic leucocytes in the peritoneal exudate. In the case of Rabbit R 3351, the blood cells were counted hourly after the injection and by 4 hours the neutrophilic leucocytes had risen from 4,860 to 11,023 per c.mm.

Thus there is an immediate draining of neutrophilic leucocytes into the peritoneal cavity but it is not lasting unless infection supervenes. Rabbit R 3351 did not show any infiltration of the tissues with eosinophiles, which subsequently becomes a constant finding.

By 4 days, all of the animals receiving the wax have shown a multiplication of monocytes around the particles of the wax, but we have not found them fusing into giant cells until the 6th day. The omentum of Rabbit R 3301, which was studied 7 days after the first injection, showed the milk spots increased in size and dense with monocytes; most of the milk spots had giant cells, some as many as thirty. All the giant cells were small because the material was introduced in a fine suspension, the size of the foreign body giant cell being proportional to the size of the mass it has engulfed. The omentum stained for acid-fast material gave a brilliant result. The acid-fast material was almost all on the milk spots in masses from about 5 to 20μ in diameter. The largest masses were surrounded by monocytes not yet fused into giant cells. All of the giant cells were filled with the acid-fast material. In sections of the omentum the giant cells showed a vacuolated cytoplasm indicating the zones from which the wax had been dissolved out in the processes of embedding. This explains why it is impossible to obtain positive acid-fast stains in sections of this material, because the free waxes are so easily removed by lipoidal solvents. The milk spots of the omentum were infiltrated with considerable numbers of eosinophilic leucocytes, but practically no neutrophilic leucocytes. In spite of this, there were no eosinophiles found free in the peritoneal exudate.

A later stage of the process is shown in Figs. 2, 3, and 4, representing 17, 27, and 32 days after the first injection. All the figures show giant cells of the omentum, photographed while the cells were living. All of the black color in the photographs represents the neutral red in the vacuoles that contain the wax. An acid-fast stain shows the wax itself; the supravital neutral red shows the fluid that the cell has secreted around the wax. It is clear that with the longer time, Fig. 3 at 27 days, much more wax has been phagocytized than was present at 17 days, Fig. 2. The reaction shown in Fig. 3 is of small tubercles of giant cells.

Three of the rabbits, R 3323, 3325, and 3326, which received the material in colloidal suspension gave suggestion of the fate of the wax within the cells.

These animals were killed 30, 32, and 46 days respectively after a single injection of the material in colloidal suspension. When the fresh omenta of these three animals were stained in the Ziehl-Neelsen technique, it was noted that *instead of the massive red staining of the giant cells seen in Rabbit R 3301, killed 7 days after the injection, the reaction had now been modified.* The giant cells of the first two animals, 30 and 32 days after the injection, all had diffuse, pink-stained zones in the center of which were tiny, bright red granules, while in the cells of the third animal, killed at 46 days, only the diffuse pink reaction remained. The type of giant cell seen in the omentum of Rabbit R 3325 is shown in Fig. 4. This cell is stained in neutral red; it is the type that showed the tiny, red granules surrounded by a diffuse, pink zone when stained for acid-fastness. It will be noted that the vacuoles of the cell, dark in the photograph, are irregular in size, but many of them, especially in the lower part of the cell, are small. In the cells of Rabbit R 3326, which had lost all of the material staining typically acid-fast, the cytoplasm of the living cells showed that the wax had become much more finely divided. Indeed, the cytoplasm reminded one of masses of platelets showing the same clumping of tiny granules.

These observations indicate that the wax becomes broken up in the cytoplasm of the giant cells until it is too finely divided to give an acid-fast stain, just as occurs when the wax is put into colloidal suspension.

In the case of Rabbit R 3323, most of the wax had flooded into the retrosternal lymph nodes, where the sinuses were distended with foreign body giant cells and the follicles had been much encroached upon by them. In these giant cells, the vacuoles were all small, giving evidence that the cells had been breaking the wax into small particles. Around these giant cells were many eosinophilic leucocytes. The omentum had so little of the reaction that the milk spots, though they showed a few giant cells, were not increased in size by them. In Rabbit R 3325, on the other hand, the milk spots were massive with giant cells.

The last three animals in the group, Rabbits R 2839, R 2845, and R 2844, killed 55, 137, and 228 days after the first injection of the wax, all had massive tubercles of foreign body giant cells in the omentum. Some of the milk spots were so thick that they were markedly raised from the surface of the omentum and some were even on pedicles. Within the giant cells there had been a progressive breaking up of the wax into smaller particles.

The type of giant cells is shown in Figs. 5 and 6 from the omentum of Rabbit R 2844. Fig. 5 is a foreign body giant cell of enormous size; the photograph is at low magnification, namely, 400 diameters. The cell has over a hundred nuclei. Smaller giant cells with from three to six nuclei are seen in the neighborhood. The cytoplasm is rather uniformly mottled, showing places from which the wax has been dissolved out in embedding. This is not as marked in sections as in the living cell on account of the shrinkage suffered during embedding.

Fig. 6 is a part of a tubercle of giant cells; again there is a great range in the size of these cells. One of the giant cells shows a central cavity from which the wax has been dissolved out. In the fresh preparations a few of the giant cells still showed unchanged wax in the center and this is evidently one of them. It will be noted in Figs. 5 and 6 that there are no signs of necrosis in any of the cells. All of the nuclei appear normal. This is characteristic of the sections throughout. In the sections of this animal there are many zones, such as the one marked with an arrow in Fig. 6, in which there is the suggestion that this type of giant cell may split into its component monocytes when the wax has become sufficiently finely divided. The history of the view that certain giant cells in tuberculous tissue do not regress by necrosis but rather break up into smaller cells was reviewed by Hektoen (7) in 1898, who brought evidence in support of this view from the study of a case of tuberculous peritonitis.

Reactions to Unsaponifiable Wax from H-37 Given Intravenously.—In Table II are given the protocols of eleven rabbits which received the unsaponifiable wax intravenously, five of which were subsequently inoculated with bovine tubercle bacilli.

The first five animals received the wax in doses of 3 mg. suspended in mineral oil and all showed foci of oil droplets in the septa between air sacs. In these zones there was an increase in monocytes which had phagocytized the oil.

Rabbit R 3350 received six intravenous injections of the wax in colloidal suspension in water and was killed 32 days after the first injection. In this manner all of the complicating factors from the menstruum were removed and the reaction to the wax came out. Throughout the liver there were many giant cells in the sinuses, two to four in every oil immersion field. There was no reaction around them; no eosinophilic leucocytes were found. Some of these giant cells had only two or three nuclei; they were simple in type, with the nuclei in the periphery and the cytoplasm vacuolated in the center. Others had as many as thirty nuclei; these were complex, having two or three cytoplasmic centers surrounded by nuclei in rings or with clumps of them along the border. In almost all of these giant cells the cytoplasm was vacuolated. In the spleen there were many small giant cells, both in the sinuses and in the follicles; many of the follicles had small, tubercle-like bodies of monocytes. Also many of the follicles had small patches of early amyloid degeneration; occasionally a follicle was almost replaced by this type of degeneration. There were no more eosinophilic leucocytes in the spleen

TABLE II

Protocols of Rabbits Receiving Unsaponifiable Wax from Tubercle Bacillus, H-37, Intravenously

Animal No.	No. and amount of injections	Time	Method of preparing material	Inoculation with bovine tubercle bacilli	Tissues
R 2001	2 3 mg.	1 wk. apart. K 24 hrs. after second injection	Dissolved in 1 cc. mineral oil	—	Many foci of oil droplets, monocytes, and neutrophilic leucocytes in septa between air sacs in lungs. Tracheal lymph nodes were filled with leucocytes and the spleen had many clasmatocytes engorged with them
R 2002	3 3 mg.	1 wk. apart. K 24 hrs. after third injection	" "	—	Foci of monocytes and leucocytes, with occasional highly vacuolated mononuclear cells in septa between air sacs in lungs. Some of these foci large enough to obliterate a few air sacs. Spleen shows much destruction of leucocytes
R 2003	4 3 mg.	1 wk. apart. K 24 hrs. after fourth injection	" "	—	Many foci of monocytes and leucocytes with some highly vacuolated mononuclear cells and a few foreign body giant cells in lungs. Some large enough to obliterate 20 air sacs. Spleen shows a few small, tubercle-like masses of monocytes
R 2004	4 3 mg.	1 wk. apart. D 7 days after fourth injection	" "	—	Larger foci of highly vacuolated mononuclear cells in lungs. These foci are vascularized and contain a few free leucocytes. Liver shows extreme involvement with coccidiosis and spleen has foci of necrosis
R 2006	6 3 mg.	1 wk. apart. K 2 days after sixth injection	" "	—	Foci of cells in lungs, 2 to 3 mm. in diameter, consisting mainly of highly vacuolated mononuclear types. A few dead leucocytes and many eosinophilic leucocytes. No destruction of leucocytes in the spleen but some foci of monocytes

R 3350	6 12 mg. 24 " 12 " 10 " 8 " 8 " — 74 "	Daily except last interval was 2 days. K 25 days after sixth injection	Colloidal suspension 12 mg. of wax per 1 cc. water	—	Many small, foreign body giant cells in sinuses throughout the liver. Many foreign body giant cells in spleen both in sinuses and in follicles. Few giant cells in septa between air sacs in lungs and in glomeruli of kidney. No infiltration with eosinophiles
R 2006 R 2007 R 2008 R 2009 R 2010	6 3 mg.	1 wk. apart	Dissolved in 1 cc. mineral oil	0.1 mg. Strain B-1, intravenously, 2 days after sixth injection	Survived an average of 176 days, with a range of 57 to 306 days, as compared with a survival of 162 days, range of 30 to 287 days for 15 controls (R 2073–R 2087). There were no differences in the type of disease in the rabbits which had received the wax in oil, but they all had foci of highly vacuolated mononuclear cells in lungs; these foci did not become invaded with the tuberculous process

than normally. An occasional small giant cell was found in the lung and also in the glomeruli of the kidney. The blood cells of this animal were studied, and from the time of the fourth injection until the animal was killed, the eosinophilic leucocytes were above 500 per c.mm., and twice they were above 1,000. The average number of eosinophiles in the normal rabbit is 110 per c.mm.

The last five animals on Chart 2 received six injections each of 3 mg. of the wax in mineral oil at intervals of 1 week and, 2 days after the last injection, were inoculated intravenously with 0.1 mg. of bovine tubercle bacilli, Strain B-1. At the same time, fifteen rabbits were inoculated with the same dose from the same suspension for controls. There were no significant differences in longevity between the injected animals and their controls, nor in the range of survival. All of the animals which had received the wax had small foci of vacuolated cells in the septa between air sacs, but these foci did not become invaded with the tubercle bacilli, resulting in setting up tubercles in them. Occasionally two or three of these foci became surrounded by tuberculous masses, but even then they were not invaded by epithelioid cells. Thus there was no sign that these abnormal foci of cells in the lungs had any effect whatever on the tuberculous infection.

DISCUSSION

The most interesting biological property of these solid alcohols and hydroxy acids which make the unsaponifiable material of Mycobacteria and contribute the property of acid-fastness to them is that, though they are stable and seemingly inert substances chemically, they are remarkable stimulants for the new growth of cells. Their essential property is that they stimulate the formation of monocytes wherever they lodge in the tissues. These monocytes then fuse around the wax and become foreign body giant cells.

It is clear that whenever lipoids are introduced parenterally, they are dealt with by the phagocytic mononuclear cells, that is, by monocytes. For this study the omentum gives the most valuable data; after intraperitoneal injection, it is easy to see that lipoids lodge on the milk spots of the omentum and not in the interspaces between them. This has been illustrated in Fig. 1 by means of the acid-fast property of the tuberculo-wax. When the lipoid is introduced in the form of a uniform colloidal suspension, we must assume that the fluid passes both through milk spots and interspaces, but only the part of the wax which floods through the milk spots becomes fixed (Fig. 1). This must be either because of the greater density of the primitive cells and monocytes which make up the milk spots, or on account of

the characteristics of their surface films. Tiny foci of young, connective tissue cells, so readily identified as milk spots in the omentum, exist throughout the connective tissues, and their property of fixing lipoids to their surface and then phagocytizing them may well be the source of the monocytes in the local formation of tubercles.

We have previously studied the effect of another lipoid especially characteristic of tubercle bacilli, namely, a phosphatide (6, 8–10), on the cells of the connective tissues. The phosphatide, in contrast to the waxes, is readily dispersed in water, making stable suspensions which are suitable for parenteral injection. In water these phosphatides form myelin-like figures (10) which can be readily identified within cells. It is thus possible to show that these phosphatides are phagocytized by monocytes and that they are acted upon within the cell in a specific manner. The material is at first irregular in size, making a cell that appears highly vacuolated in fixed material, since the processes of treating tissues for sectioning result in a complete solution of the lipoid. However, it is soon broken up into small and then smaller particles within the cytoplasm. This process seems to go on uniformly so that the particles are all about the same size at any stage, except immediately after phagocytosis. When they have become finely divided, the cell is the typical epithelioid type, indistinguishable from the form seen in the disease tuberculosis. The time necessary for the formation of epithelioid cells ranges from 4 days to 2 weeks. Some of the monocytes which have phagocytized the phosphatide become multinuclear, making Langhans' giant cells.

The only constituent of the phosphatide capable of bringing about this reaction is a fatty acid, discovered by Anderson and named by him phthioic acid. This acid is optically active and has the formula of $C_{26}H_{52}O_2$. It has not been found in nature before and is highly characteristic of tubercle bacilli. It occurs in all of the three major lipoid fractions as first obtained through solvents, namely, in the alcohol-ether-soluble phosphatide, in the acetone-soluble mixture of fatty acids, and in the chloroform-soluble material (1, 11–13). When this fatty acid is given either in the form of salts (5), or with its burning properties cut down by suspension in nujol, it forms typical epithelioid cells, singly and in tubercles (8–10).

The fate of the epithelioid cell is an essential point in judging its

meaning; after the introduction of the lipoid either as phosphatide or as fatty acid, it is broken up into fine particles, after which no further change can be detected in the cell, except that refractive bodies may appear in the periphery. The end-result, though it may be long delayed, is the death of the cell. Thus the monocyte can readily phagocytize this material and disperse it to a certain state, but it seems not physiologically adapted to a disintegration of this fatty acid into its simpler molecular groups. Epithelioid cells then regress, either through the death of the individual cells, or *en masse*, in which case the phenomenon is called caseation (10).

The giant cells produced in response to the waxes must be compared with the epithelioid types. The unsaponifiable material from H-37, which is a higher hydroxy acid with the formula $C_{94}H_{188}O_4$, can be identified within the cells by the property of acid-fastness. Only the finest particles of this material, as when introduced in colloidal suspension, are phagocytized by the individual monocyte; rather the masses of the wax become surrounded by many monocytes which fuse into a giant cell which is at first a hollow sphere. The large inner surface of this fused cytoplasm is then able to engulf the material.

When the wax is sufficiently finely divided, as in the colloidal suspension, it gives only a diffuse pink reaction with the Ziehl-Neelsen technique, but when the small particles again become aggregated, typical acid-fastness is restored. This property gives an opportunity to follow the action of the giant cells upon the wax. Whether the material has been introduced in colloidal suspension or in powder form, it appears within a period of 2 to 3 weeks as massive aggregates which are acid-fast. The material remains for a long time within the giant cell in the form of irregular masses, with no immediate tendency toward the formation of particles of equal size, so characteristic of the epithelioid type. But in the period of months, typical acid-fastness gradually grows less, until only a diffuse, pink reaction like that of the colloidal suspension remains. This indicates that the cell is able to disperse this material into finer particles, such as are in the colloidal suspensions. In sections the giant cells which were originally coarsely vacuolated become much more finely vacuolated, as is shown in Figs. 5 and 6. If such a giant cell is small and has peripheral nuclei, as is shown in Fig. 5, it is indistinguishable from the typical Langhans' type.

Thus the method of dealing with the wax within the giant cells is different from that of dealing with the phosphatide. It is a slower process and there is nothing that might be compared to a process of emulsification as with the phosphatide, but there is a gradual disintegration of the material until it is too finely divided to give an acid-fast stain.

More important still is the difference in the fate of the cell which has taken in the wax. We have not detected any signs of damage to the cells; they do not disappear through caseation; there is no sign of cell death in the tissues. It is probable that the giant cells eventually separate into single monocytes, though our experiments have not been carried far enough to prove this point.

The first reaction to the introduction of the wax into the peritoneal cavity is the calling of neutrophilic leucocytes into the peritoneal cavity, but this follows the introduction of any foreign material whatever. It is a reaction which does not persist, and in about 2 weeks' time the neutrophilic leucocytes have disappeared from the tissues and eosinophilic leucocytes appear. This reaction is so marked that it can be detected by an increase in eosinophilic myelocytes in the bone marrow and, if the blood cells are followed, by an increase in their number in the blood stream. They do not seem to wander into the peritoneal exudate but rather remain around the giant cells. Their presence is so constant around these lesions that they suggest that the giant cells break some material from the wax which is chemotaxic to eosinophiles. Some of the foci of giant cells, especially those that form quite large tubercles, also become surrounded or infiltrated with lymphocytes. It is not a constant reaction, tubercles of giant cells in any one section varying as to whether there are few, many, or no lymphocytes around them.

The study of the effect of the unsaponifiable material injected intravenously on the course of a subsequent infection with tubercle bacilli indicates that the waxes have no effect on resistance. These materials are elaborated by the Mycobacteria and give them the property of acid-fastness by which they are classified, but their function is probably in connection with the life and the survival of the bacillus and they induce no reactions in the host that are associated with resistance.

CONCLUSIONS

1. The unsaponifiable fractions of the Mycobacteria, though insoluble in water and extremely stable chemical compounds, are nevertheless remarkable stimulants of cells.

2. They give rise to new monocytes which surround these waxes and then fuse into giant cells which engulf them.

3. The property of acid-fastness of the waxes makes it possible to identify them within the giant cells which have phagocytized them.

4. Within the foreign body giant cells the waxes are slowly disintegrated. They appear not to damage the cells which engulf them, and hence one may infer that they take no part in caseation.

5. They have no effect on the resistance of the host.

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EXPLANATION OF PLATES

PLATE 32

FIG. 1. Film of omentum of Rabbit R 3351, which had received one intraperitoneal injection of 24 mg. of the unsaponifiable material from human tubercle bacilli, Strain H-37, suspended in 2 cc. of water. Killed 24 hours after the injection. Film stained with the Ziehl-Neelsen technique to bring out acid-fast material which shows as black masses against the milk spots. × 10.

FIG. 2. Foreign body giant cell in the center of a milk spot of the omentum, photographed while the cells were living, of Rabbit R 2814, which had received two intraperitoneal injections of the unsaponifiable material from the human

tubercle bacillus, H-37, and was killed 14 days after the second injection. The material was introduced as a dry powder through an incision under anesthesia. The film is stained with neutral red and was photographed while the cells were still living. The material stained black represents the fluid around the particles of wax, which had been phagocytized; there is only a small amount of this reaction within the giant cell; there are well stained monocytes in the border. The refractive bodies seen around the giant cell are in the slightly stimulated serosal cells covering the milk spot. × 1040.

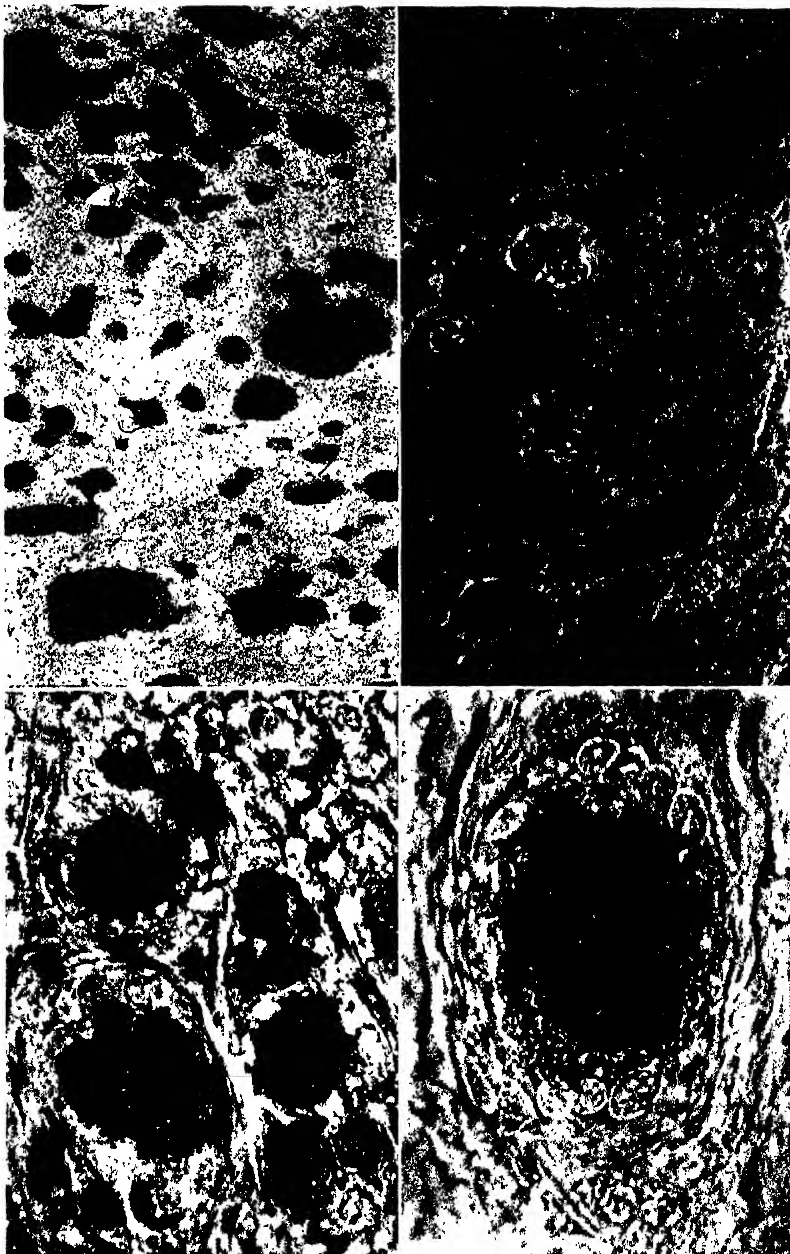
FIG. 3. Small tubercle of foreign body giant cells in a film of omentum of Rabbit R 2806, which had received two intraperitoneal injections of the unsaponifiable material from the human tubercle bacillus, H-37, and was killed 23 days after the second injection. The material was introduced as a dry powder through an incision under anesthesia. The film is stained with neutral red and was photographed while the cells were still living. The material stained black represents the fluid around the particles of wax, which had been phagocytized, and shows a marked increase from the stage of Fig. 2. The wax is in relatively large masses. × 693.

FIG. 4. Foreign body giant cell in a film of omentum of Rabbit R 3325, which had received one intraperitoneal injection of the unsaponifiable material from human tubercle bacillus, H-37, and had been killed 32 days later. The material was introduced in colloidal suspension in 3 cc. of water. The film was stained with neutral red and photographed while the cells were still living. The material which shows as black (neutral red) represents the fluid secreted by the cell around the particles of the wax and indicates that some of the wax is still in large aggregates while some is in fine particles. × 866.

PLATE 33

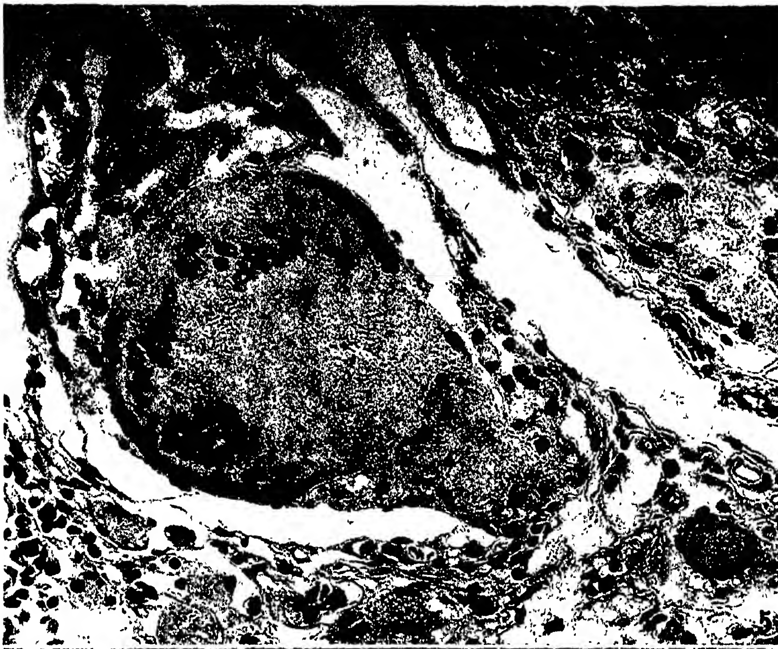
FIG. 5. Large and small foreign body giant cells in a section of the omentum of Rabbit R 2844, which had received two injections of the unsaponifiable material from the human tubercle bacillus, H-37, and had been killed 222 days after the second injection. Stained with hematoxylin and eosin. × 400.

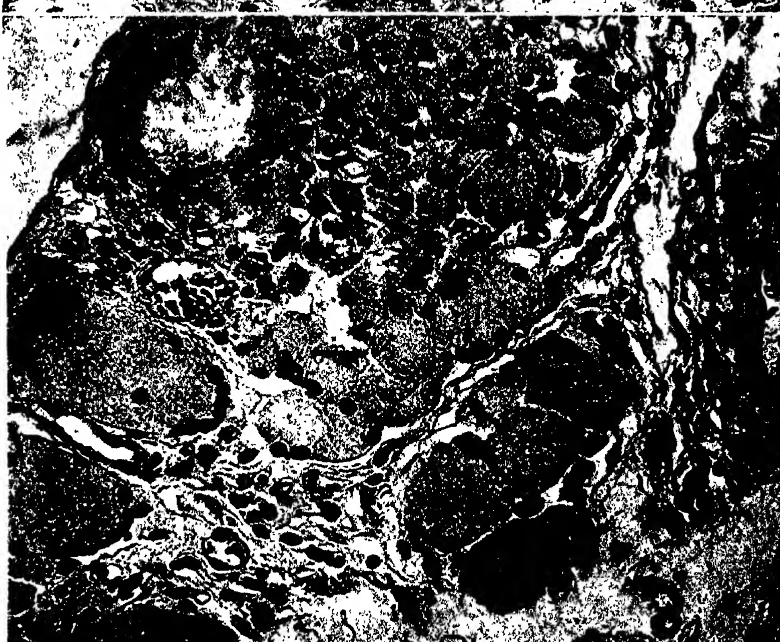
FIG. 6. Tubercles of foreign body giant cells in a section of the omentum of the same animal as in Fig. 5. The arrow points to a zone in which a giant cell may be breaking up into its component monocytes. Stained with hematoxylin and eosin. × 400.



Photographed by Louis Schmidt

(Sabin *et al.*: Wax-like materials from acid-fast bacteria)





Photographed by Louis Schmidt

('abin *et al.*: Wax-like materials from acid-fast bacteria)

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CELLULAR REACTIONS TO WAXES FROM MYCOBACTERIUM LEPRAE

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Plate 34

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The plan for chemical analysis of the Mycobacteria sponsored by the National Tuberculosis Association included an acid-fast strain isolated from a case of leprosy, as well as selected strains of human, bovine, and avian tubercle bacilli, and this study deals with the waxes extracted from *Mycobacterium leprae*.[1] The chemical analyses have not yet been completely reported (1, 2). The materials used have been in different stages of purification and have therefore provided the opportunity to compare the complex cellular reactions to mixtures of substances with the simple response to highly purified crystalline alcohols.

Materials and Methods

The strain of organisms used for the analysis was obtained from a case of leprosy (Apa case) in Honolulu, about 1909. It has been kept at the Hygienic Laboratory, Washington, D. C., as Strain 370; on Feb. 4, 1926, a subculture was given to the Mulford Biological Laboratories, Glenolden, Pennsylvania. It was there grown on Long's synthetic medium in quantities adequate for chemical analysis.

Five preparations of wax fractions from the *Bacillus leprae* have been studied.

1. A Crude Chloroform-Soluble Wax.—This was obtained in the same manner as the corresponding fraction from tubercle bacilli (3). This fraction has not yet been analyzed completely, but Dr. Anderson reports that it is almost free from phosphorus and nitrogen and hence is not a phosphatide (1). It is a red, wax-like substance, looking like beeswax except for the color.

2. A Crude Wax Obtained from the Purification of the Lepra Phosphatide.—This material could be removed from the crude phosphatide because it is more

[1] We are indebted for this material to Dr. R. J. Anderson and his associates at Yale University, who extracted the waxes from *Mycobacterium leprae*.

soluble in a mixture of ether and acetone than the phosphatide, and it came out on cooling the acetone solution (1). Concerning this product, Dr. Anderson has written us, "This product consists mainly of a solid glyceride which I call 'Leprosin.' It contains a number of fatty acids and the alcohols $C_{20}H_{42}O$ and $C_{18}H_{38}O$, identical with the alcohols isolated from the wax of the timothy grass bacillus." This material is a slightly pink, amorphous powder; that is, it carries some of the pigment so characteristic of the *B. leprae.*

3. Leprosin.—This was obtained from the purification of the preceding material. Though a glyceride instead of an alcohol, it corresponds in a general way in its physical properties to the unsaponifiable material from tubercle bacilli. It is a snow white powder, with a melting point of 54°C. It is optically active.

$$[\alpha]_D^{23} \text{ in chloroform } + 4.2°.$$

4. Leprosinic Acid.—This was obtained from the analysis of the leprosin and in physical properties is like it. It has a melting point of 61–62°C. and is dextrorotatory:

$$[\alpha]_D^{23} \text{ in chloroform } + 4.7°.$$

All four of these materials from the *B. leprae* are acid-fast.

5. Alcohol II from the Unsaponifiable Matter of the B. leprae.—This is a pure white material consisting of feather-like crystals. It has a melting point of 84°C. and a formula of $C_{25}H_{44}O_2$. It is not acid-fast.

Besides this alcohol from the *B. leprae* we have also received from Dr. Anderson a similar material as follows:

Alcohol Isolated from the Wax of the Timothy Grass Bacillus.—This is a pure white material made up of fine, needle-like crystals. Its melting point is 63°C. It is dextrorotatory:

$$[\alpha]_D^{21} \text{ in ether } + 6.93°.$$

Its formula is $C_{20}H_{42}O$. It is not acid-fast.

None of these preparations has been given in nujol, the method used in our first study of wax fractions from tubercle bacilli (4). Rather it has been found better to introduce these materials in the form of a dry powder through an incision under ether anesthesia. The crude waxes can be ground into a fine powder if they are first chilled with dry ice until brittle. The more purified substances, the leprosin, the leprosinic acid, and the alcohols, do not need more than simple grinding to separate the clumps of crystals.

The method of preparing these waxes from the *B. leprae* in colloidal suspension, which was developed by one of us with the unsaponifiable material from the tubercle bacillus (5), was tried with the lepra fractions but with less success. It consisted of dissolving the wax in chloroform and adding an equal amount of hot alcohol. To this solution the same amount of distilled water was added drop by

drop, which procedure threw down the wax in the form of a precipitate. The suspension obtained from the crude chloroform-soluble wax was not as fine as that from the unsaponifiable material from the tubercle bacillus but rather consisted of particles about 7μ in diameter which settled out into quite large aggregates. This material, therefore, had to be freshly prepared for each injection.

When the crude wax obtained from the purification of the lepra phosphatide was prepared for colloidal suspension, the precipitate was of more or less regular pentagonal particles, highly refractive and suggesting a crystalline form. There was no agglutination of these particles.

In attempting to prepare similar suspensions of leprosin and leprosinic acid, the addition of distilled water resulted in the separation of minute droplets of an oily nature which formed an unstable emulsion. This emulsion had a tendency to curdle and adhere to the surface of the flask. This was in marked contrast to the stability of the suspension prepared from the unsaponifiable material from H-37.

RESULTS

Reactions to the Crude Chloroform-Soluble Wax from B. leprae.—The crude chloroform-soluble wax from the *B. leprae* was given intraperitoneally to six rabbits, as shown in Table I.

'Rabbits R 4268 and R 4269 both received the material after it had been powdered with dry ice. The material did not give a simple reaction; where the wax had lodged, the area became infiltrated with leucocytes making an abscess. Around these abscesses were monocytes, fibroblasts, and clasmatocytes, filled with leucocytes. In the omentum the reaction was not uniform; there were abscesses, bands of fibroblasts, and foci of monocytes, some showing partial fusion into giant cells. There was a marked dilatation of the vessels. The retrosternal lymph nodes had their sinuses filled with monocytes and in some places they were present also in the follicles.

The same tendency toward the formation of abscesses followed the introduction of the material in colloidal suspension.

This preparation showed a marked tendency to a clumping of the particles, with the result that the dose varied; Rabbit R 4189 received a small dose, R 4187 an average dose, and R 4188 a massive dose. The abscesses were smaller because the masses of the wax had been smaller. They were filled with clasmatocytes containing leucocytes. Around the abscesses were monocytes and giant cells, many of them vacuolated, indicating a phagocytosis of the wax. The edge of one of the abscesses with a narrow border of giant cells is shown in Fig. 1, from Rabbit R 4188. Bands of fibroblasts were extensive; in one animal of the group, R 4188, there was an extreme involvement of the retrosternal lymph nodes with

TABLE I

Protocols of Rabbits Which Received the Crude Chloroform-Soluble Wax from B. leprae

Animal No.	No. and amount of injections	Time	Method of preparing material	Peritoneal exudate: Percentage of PMN	Lymph.	Mono.	Tissues
		days					
R 2795	1 20 mg.	4 K	Dry powder through cannula	—	—	—	Most of the wax in one bolus. Coccidiosis present
R 4268	1 50 mg.	6 K	Material powdered with dry ice and introduced through incision under anesthesia	15.0	14.0	70.80	Many abscesses, 2 to 3 cm. in diameter in omentum, on cecum, in the peritoneal wall, and in incision. Abscesses surrounded by monocytes and fibroblasts, with clasmatocytes containing leucocytes in the border. Omentum almost too massive to study as film and very complex: zones of fibroblasts; many small foci of monocytes showing partial fusion into giant cells, the center of which contain leucocytes. Marked vascular dilatation. Retrosternal nodes have sinuses filled with monocytes
R 4269	1 50 mg.	6 K	" "	63.68	0	36.31	Many abscesses 2 to 3 cm. in diameter in omentum surrounded by monocytes, fibroblasts, and clasmatocytes containing leucocytes. Many small foreign body giant cells with vacuolated cytoplasm. Diaphragm shows tubercles of giant cells in which there is only partial fusion of the monocytes. Leucocytes infiltrating the tissues. Bands of fibroblasts; marked dilatation of the vessels. Extensive reactions of the same type in retrosternal nodes replacing part of the follicles

R 4187	3 20 mg.	4 4 K 5 days after third injection	Colloidal suspension	1.78	22.19	76.02	No symptoms. No rise in temperature. Small abscesses in body wall surrounded by fibroblasts and clasmatocytes containing leucocytes. Omentum has no large abscesses but many small tubercles of foreign body giant cells and monocytes, some vacuolated. In the center of the tubercles were leucocytes. Dilatation of the vessels and marked bands of fibroblasts. Few giant cells in retrosternal lymph node. One small hemorrhage in one lung, infiltrated with monocytes
R 4188	3 20 mg.	4 4 K 5 days after third injection	" "	8.88	24.36	66.75	No symptoms. No rise in temperature. Abscesses 4 to 10 mm. in diameter on surface of cecum, in capsule of liver and spleen, on diaphragm, in body wall, and in the omentum. Abscesses contain clasmatocytes filled with leucocytes and are surrounded by a narrow band of monocytes. Omentum has many small tubercles of foreign body giant cells and monocytes, some of them vacuolated. Dilatation of vessels and bands of fibroblasts. Lungs had several small, translucent nodules which were made up of monocytes. Retrosternal nodes show extreme involvement with monocytes and giant cells both in the sinuses and in the follicles. In the fresh tissue, typical epithelioid cells were seen, confirmed in sections
R 4189	3 20 mg.	4 4 K 5 days after third injection	" "	0.25	42.45	57.28	Very slight reaction; a few abscesses in the body wall and a few foreign body giant cells in the omentum

TABLE II

Protocols of Rabbits Which Received the Wax Obtained in the Purification of the Phosphatide of B. leprae

Animal No.	No. and amount of injections	Time	Method of preparing material	Peritoneal exudate: Percentage of PMN	Peritoneal exudate: Percentage of Lymph.	Peritoneal exudate: Percentage of Mono.	Tissues
		days					
R 2715	1 20 mg.	3 K	Dry powder through cannula blown in with air	1.02	5.10	93.87	Omentum showed increased size of milk spots with particles of wax on them. Increase in monocytes; occasional fusion into giant cells, some having as many as 50 nuclei. Few epithelioid cells. Infiltration with neutrophilic leucocytes and a few myelocytes. No increase in fibroblasts. Retrosternal node has many monocytes in the sinuses
R 2800	1 20 mg.	4 K	Dry powder through incision under anesthesia	1.13	1 13	97.72	Omentum showed increased size of milk spots with particles of wax on them. Around the particles were young monocytes showing no signs of phagocytosis; that is, no vacuoles staining with neutral red. Few neutrophilic and eosinophilic leucocytes. No increase in fibroblasts. Eosinophilic myelocytes prominent in the bone marrow
R 2798	2 20 mg.	4 D	Dry powder through cannula. Died after second injection which punctured the liver	—	—	—	Wax in single bolus and reaction slight. Few foreign body giant cells in omentum. Blood in retrosternal lymph nodes 5 min. after puncture of the liver

R 2763	2 20 mg.	4 K 3½ hrs. after second injection	Dry powder through cannula blown in with air	Cells all dead			3 hrs. after the second injection the animal had a convulsion, was extremely sensitive on being touched, became cyanotic, pulse was about 250 and respiration was slow. Temperature was 111.5° and the animal was killed. Small abscesses in mesentery, diaphragm, and omentum. Omentum showed increased density of the milk spots with monocytes and giant cells, dilatation of the vessels, neutrophilic leucocytes, both free and in clasmatocytes, and a few epithelioid cells. Marked damage of the lymphocytes in the nodes, probably due to the temperature. Congestion of the lungs and liver. Hemorrhage in the bone marrow
R 4184	3 20 mg.	4 4 K 6 days after the third injection	Colloidal suspension in 4 cc. water	5.59	45.29	49.10	No symptoms; no rise in temperature. Small abscesses on peritoneal wall near places of injections, on the large intestine, on the diaphragm, and in the omentum. Milk spots of omentum increased in size and number. Abscesses of omentum contain many clasmatocytes filled with leucocytes and have a wide border of giant cells. Many giant cells in tubercles and diffusely scattered. Marked vascularization, bands of fibroblasts, and increase in fibrous tissue. Acid-fast stain negative. Retrosternal nodes show extreme numbers of monocytes in the sinuses and the follicles are almost replaced by them. Small, translucent nodules in lungs made up of monocytes

TABLE II—*Concluded*

Animal No.	No. and amount of injections	Time	Method of preparing material	Peritoneal exudate: Percentage of PMN	Peritoneal exudate: Percentage of Lymph.	Peritoneal exudate: Percentage of Mono.	Tissues
		days					
R 4185	3 20 mg.	4 4 K 6 days after the third injection	Colloidal suspension in 4 cc. water	27.62	18.31	54.05	No symptoms. No rise in temperature. Lesions like those of R 4184, except less formation of fibers in the omentum and less reaction in the retrosternal nodes. Nodules of monocytes in the lungs
R 2771	3 20 mg.	4 4 K 5 days after the third injection	Dry powder through cannula	5.20	13.54	81.25	No symptoms after the first injection. After the second, rapid respiration and temperature fell 2.1° and then rose 3.3°. After the third the temperature fell 2.5° and then rose 4.8°. One of the injections had lodged in the sheath of the external oblique muscle, where there was a nodule of giant cells, and monocytes, many of them vacuolated; very few neutrophilic leucocytes but many eosinophilic. Omentum showed increased density of the milk spots due to monocytes and giant cells, whose cytoplasm was filled with vacuoles. Some wax still to be seen on the milk spots. Some typical epithelioid cells. Moderate increase in fibroblasts

monocytes and giant cells, as shown in Fig. 3 which is a section through the sinuses. The follicles of this node were also extensively replaced by similar cells. Besides these reactions, typical epithelioid cells were found in the omenta and the retrosternal lymph nodes of these animals, both with the supravital technique and in sections.

Reactions to Wax from the Purification of the Phosphatide of B. leprae.—The wax obtained from the purification of the lepra phosphatide was given to seven rabbits, as shown in Table II. This was the only material given which was followed by any symptoms and they proved to be inconstant.

The early reaction was shown by two rabbits, R 2715 and R 2800, one of which received the material through a cannula and the other through an incision. In neither animal were there symptoms after the injection. The wax had lodged on the milk spots of the omentum, and around the particles of the wax there had been a multiplication of monocytes; in places these monocytes had started to fuse into giant cells. There were occasional epithelioid cells but there was no increase in fibroblasts.

Rabbit R 2763 showed extreme disturbance after the second injection, 3 hours later it had a convulsion, the temperature rose to 111.5°, and the animal was therefore killed. A second rabbit, R 2771, had a rise in temperature of 3.3° after a third injection but survived. We did not determine the cause of the fever in Rabbit R 2763. The material injected showed no bacteria. The brain and cord were normal; there were small abscesses in the omentum, in the mesentery, and on the diaphragm, but not as many as in the rabbits of the preceding series. There was the same reaction of monocytes and giant cells around the wax which had lodged on the milk spots of the omentum.

Rabbit R 2771 showed similar cellular reactions, complex in type; there were foreign body giant cells around the particles of wax, a few neutrophilic leucocytes, and many eosinophilic leucocytes. There were epithelioid cells and an increase in fibroblasts.

The material was then given to two rabbits, R 4184 and R 4185, in three doses in colloidal suspension. None of these injections caused symptoms and there was no rise in temperature. In making the suspension, the material had been heated. There were small abscesses wherever the wax had lodged. The abscesses had wide borders of monocytes and giant cells, as is shown in Fig. 2. The width of the border of giant cells can be seen to be greater than in Fig. 1, which was taken from an animal which had received the crude chloroform-soluble wax. Indeed, almost the entire section shown in Fig. 2 is of monocytes except the abscess on the lower border. Besides this, the material from Rabbit R 4184 showed many large masses of giant cells, some in tubercles and some diffusely scattered, so that when the total reaction was compared with that in the animals

TABLE III

Protocols of Rabbits Receiving Leprosin

Animal No.	No. and amount of injections	Time	Method of preparing material	Peritoneal exudate			Tissues
				Percentage of			
				PMN	Lymph.	Mono.	
		days					
R 2801	2 20 mg.	4 K 4 days after second injection	Dry powder through incision under anesthesia	6.5	5.0	88.0	No symptoms after either injection. Omentum shows marked increase in size of milk spots with foreign body giant cells enclosing the wax. Marked bands of fibroblasts growing as in tissue culture. No epithelioid cells. Eosinophilic leucocytes in the retrosternal lymph nodes
R 4264	1 50 mg.	7 K	Colloidal suspension	5.0	13.50	80.75	In omentum the predominant reaction is an extensive formation of tubercles of foreign body giant cells. Some have hollow centers and dense cytoplasm; others have a vacuolated cytoplasm. A few abscesses in the omentum, with walls dense with giant cells. Except for these abscesses very slight infiltration with neutrophilic leucocytes. Increase in fibrous tissue. Abscess in body wall near site of injection with monocytes and giant cells in its border. Retrosternal nodes show an extensive infiltration of sinuses and follicles with foreign body giant cells and many eosinophilic leucocytes
R 4265	1 50 mg.	7 K	" "	0	3.20	96.66	Reaction slight; a few milk spots of the omentum showed wax with foreign body giant cells. One giant cell with four nuclei found in the peritoneal exudate

R 2812	2 20 mg.	4 K 14 days after the second injection	Dry powder through incision under anesthesia	—	—	—	No symptoms. Omentum shows foreign body giant cells singly and in tubercles. The giant cells have enclosed the wax and a few leucocytes. Very little vacuolization of the cytoplasm of the giant cells. Increase in fibroblasts and in bands of fibers. A few small abscesses, but almost no neutrophilic leucocytes elsewhere. No eosinophilic leucocytes
R 2807	2 20 mg.	4 K 24 days after the second injection	" "	0	9.64	90.35	Every milk spot of omentum has foreign body giant cells, some extremely large. Their cytoplasm is markedly vacuolated. Tubercles of giant cells seen in section. Many bands of fibroblasts and many eosinophilic leucocytes in the omentum. Many eosinophiles in the retrosternal nodes

that received the crude chloroform-soluble wax, it was clear that this preparation had a much greater proportion of the factor, that is the wax, that produces the foreign body giant cells. Besides the giant cells there was a marked dilatation of the blood vessels, seen also in Fig. 2, as well as signs of the new formation of fibroblasts and of fibrous tissue.

Reactions to Leprosin.—Five rabbits have received the leprosin and their protocols are given in Table III. The formation of abscesses, so marked a feature of the reaction to the two preceding materials, was much reduced, appearing only in Rabbit R 4264.

There were two constant reactions to the material, an extensive formation of foreign body giant cells and a marked development of bands of fibroblasts. These bands are shown in Fig. 4, from the omentum of Rabbit R 2801. The photograph was taken from a fresh film, stained with neutral red, and was made while the cells were living. It will be noted that the bands of fibroblasts are growing in a manner to simulate a tissue culture. The giant cells occurred singly and in tubercles and had eosinophilic leucocytes around them. No epithelioid cells were seen after the injection of the leprosin.

Reactions to Leprosinic Acid.—The cellular reactions to the intraperitoneal injections of leprosinic acid are shown in five rabbits, the protocols of which are given in Table IV. In every instance the reaction to this material has been of a single cell type; namely, there has been a multiplication of monocytes around the particles of the wax and their fusion into giant cells.

In the omentum it was clear that this material also lodged only on the milk spots which became tubercles of giant cells. The differential counts of the cells of the peritoneal exudates of these animals show how small a part of the reaction is made by neutrophilic leucocytes, for only in the first animal of the series were there any of these cells and in that instance, only 2.5 per cent. Instead the tissues were infiltrated with eosinophilic leucocytes.

Reactions to Alcohols from the Waxes of the B. leprae and B. phlei.—These two highly purified, crystalline alcohols, $C_{25}H_{44}O_2$, from the unsaponifiable wax material of the *B. leprae*, and $C_{20}H_{42}O$, from the *B. phlei*, or timothy grass bacillus, were each given intraperitoneally to two rabbits in doses of 15 mg. They were given in each instance as the dry crystals through an incision under ether anesthesia.

Rabbits R 4272 and R 4241 each received the alcohol from the *B. leprae* and were killed in 5 days. Rabbit R 4236 and R 4238 received the alcohol from the

TABLE IV

Protocols of Rabbits Receiving Leprosinic Acid

Animal No.	No. and amount of injections	Time	Method of preparing material	Peritoneal exudate, Percentage of: PMN	Lymph.	Mono.	Tissues
		days					
R 2769	1 20 mg.	5 K	Dry powder through cannula	2.52	7.07	90.40	Omentum showed accentuated milk spots with wax enclosed in giant cells. Tubercles of foreign body giant cells on diaphragm infiltrated with eosinophilic leucocytes. Many eosinophiles in the retrosternal lymph nodes
R 4271	1 30 mg.	22 K	Dry powder through incision under anesthesia	0	14.81	85.18	Omentum showed flecks of wax on the milk spots surrounded by foreign body giant cells. These giant cells were not as large as in R 4270 but there were as many as 30 to 40 on some milk spots. Foci of monocytes in septa of lungs and some giant cells in the retrosternal lymph nodes
R 4270	1 30 mg.	23 K	" "	0	13.40	86.59	Omentum showed flecks of wax on the milk spots surrounded by foreign body giant cells. Some of them had only three or four nuclei; others were so large that they completely filled a low power field. Many monocytes; no leucocytes seen. No foci of monocytes found in the lungs
R 4266	1 40 mg.	37 K	Imperfect colloidal suspension	0.51	15.97	83.50	Omentum showed many giant cells on the milk spots, some with 30 to 40 nuclei. There were 2.5% eosinophilic leucocytes in the peritoneal exudate and there was one giant cell about 100μ in diameter. A focus of giant cells and monocytes near the point of injection was infiltrated with eosinophilic leucocytes. A few foci of monocytes in the lungs and extensive involvement of both sinuses and follicles of retrosternal lymph nodes with monocytes
R 4267	1 40 mg.	38 K	" "	0	14.79	85.20	Less reaction than in R 4266 but of same type. Monocytes and foreign body giant cells containing wax on the milk spots of the omentum

timothy grass bacillus and were killed in 4 and 6 days respectively. The reaction was of giant cells around the crystals of alcohol; these crystals lodged on the milk spots of the omentum. The giant cells were proportional to the size of the crystals, whether they occurred singly or in a clump. There were more neutrophilic leucocytes in the peritoneal fluid than when the time interval had been longer, ranging from 5 to 11 per cent, except in the case of Rabbit R 4238, in which there was an adhesion of a part of the omentum to the peritoneal wall and to the liver, and the leucocytes were 39.08 per cent.

These experiments have not been completed; they involve a study of the reactions to these materials in the tuberculous rabbits, as well as in the normal ones.

DISCUSSION

The materials from the lepra bacillus have given an interesting opportunity to follow the chemical separation of complex mixtures by cells instead of in the test tube. It is clear that all of these materials contain some substance which, like the solid alcohol $C_{25}H_{44}O_2$, gives the formation of the simple foreign body giant cell. The crude chloroform-soluble wax and the wax obtained from the purification of the lepra phosphatide contain, however, many more substances that give cellular reactions. With both of them the most striking phenomenon is the formation of large abscesses wherever the masses of the wax lodge. Thus there is some substance chemotaxic to leucocytes in or on the wax which acts like the tuberculo-polysaccharide. That is, this material calls leucocytes from the blood stream and so damages them that they are readily engulfed by clasmatocytes. The leucocytes are found in clasmatocytes both in the local lesions and in the spleen. These abscesses are not at all like caseation for there is no basis of dead cells, but rather it is the wax itself which becomes infiltrated with leucocytes.

Besides this reaction, there is the formation of the foreign body giant cells with the fused monocytes enclosing both the wax and the leucocytes. In the case of the wax obtained from the analysis of the lepra phosphatide, this reaction is much greater in amount than with the crude chloroform-soluble wax, indicating that the wax-like material is in much greater proportion in this fraction. The tissues also become infiltrated with eosinophilic leucocytes and lymphocytes.

These materials also are irritants, causing a marked dilatation of the blood vessels and probably a new growth of vessels. They also contain substances that are remarkable stimulants for the formation of fibroblasts and of new fibers. Besides all of these properties, they contain some material that gives rise to typical epithelioid cells, exactly like the reaction to the tuberculo-phosphatide. Thus these two materials give reactions as complex as those aroused by the acetone-soluble material from the tubercle bacillus (6). The cellular reactions to them are similar to those aroused by the crude wax which can be separated from tubercle bacilli. These complex cellular reactions reflect the fact that some of all the different types of lipoids come out with the first use of lipoidal solvents on mixtures.

The leprosin is an entirely different type of material; it is a pure white powder in crystalline form which does not give the cellular reactions characteristic of complex mixtures of lipoids, such as have just been described. Rather its reactions are reduced to two simple properties: It causes a remarkable new growth of fibroblasts, as is shown in Fig. 4, making them grow as they do in tissue cultures. Then it produces the formation of foreign body giant cells around the particles of the wax, and subsequently gives an infiltration of the tissue with the eosinophilic leucocytes. With the leprosinic acid the material which causes the new growth of the fibroblasts has been split off from the molecule of the leprosin, and the resulting fraction causes only the formation of the foreign body giant cells. This property is seen in its purest state with the crystalline solid alcohol, in which each giant cell is proportional in size and shape to a single crystal or a clump of crystals which may happen to lodge on a milk spot.

CONCLUSIONS

1. The waxes from the *B. leprae*, like those from tubercle bacilli, are remarkable stimulants of cells.
2. The crude wax separated from the *B. leprae* is a mixture of lipoids and other materials, and gives reactions that include the types of cells characteristic of the response to the tuberculo-polysaccharide, phosphatide, and wax.
3. The wax obtained from the purification of the lepra phosphatide shows similar cellular reactions but with a greater proportion of foreign body giant cells.

4. Leprosin, though a glyceride, corresponds in its physical properties to the unsaponifiable material from the tubercle bacillus. It stimulates two strains of cells, fibroblasts and monocytes. The monocytes fuse into foreign body giant cells to engulf the wax.

5. The cellular reaction to the leprosinic acid and to the crystalline alcohols is of one type only, represented by the foreign body giant cell.

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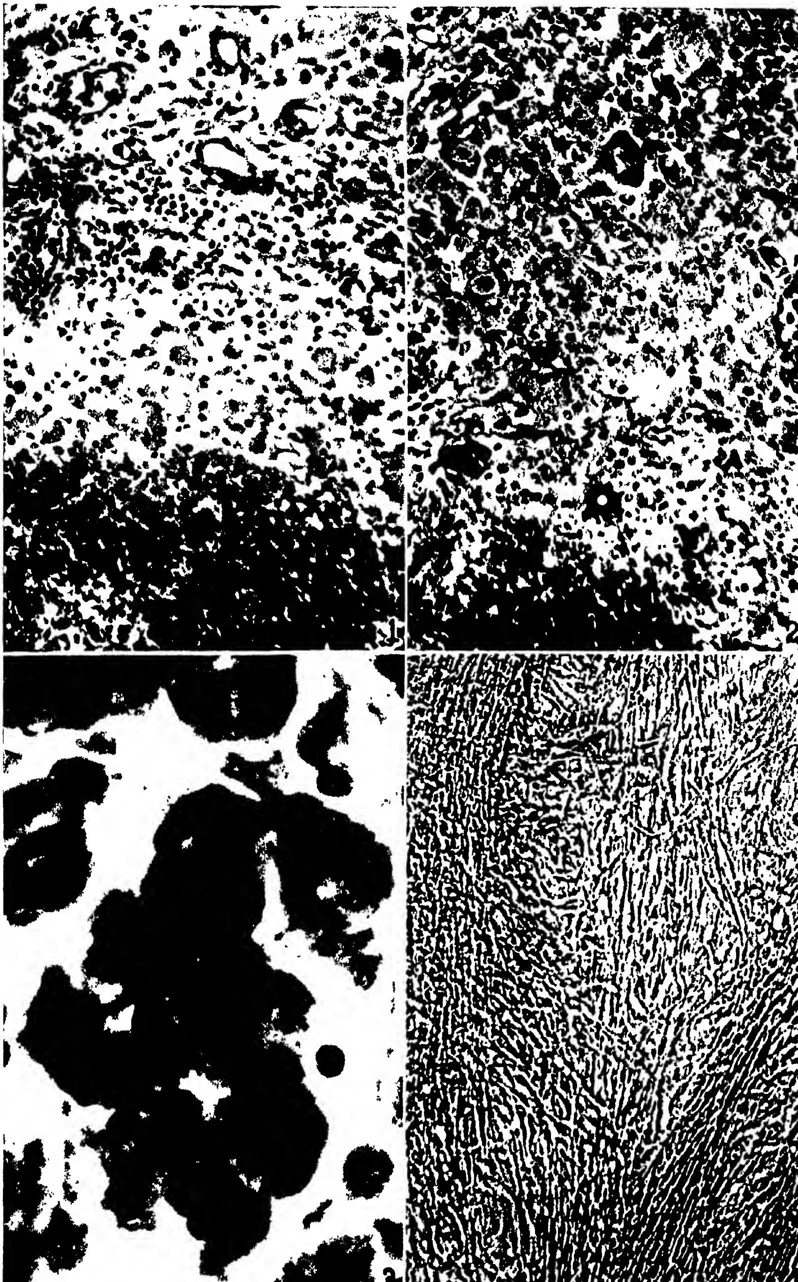
EXPLANATION OF PLATE 34

FIG. 1. Wall of an abscess in the omentum of Rabbit R 4188, which had received three intraperitoneal injections of 20 mg. each of the crude chloroform-soluble wax from the *B. leprae*. The wax was in the form of a colloidal suspension. The animal was killed 5 days after the third injection. There is a narrow band of monocytes and giant cells along the edge of the abscess. Masson stain to show the deep basophilia of the cytoplasm of the monocytes. × 210.

FIG. 2. Wall of an abscess in the omentum of Rabbit R 4184, which had received three intraperitoneal injections of 20 mg. each of the wax obtained from the purification of the lepra phosphatide. The animal was killed 6 days after the third injection. There is a wide band of monocytes and giant cells along the edge of the abscess. Masson stain. × 250.

FIG. 3. Sinuses of one of the retrosternal lymph nodes of the same animal as Fig. 1. It shows a pure and extensive reaction of monocytes and giant cells. Masson stain. × 1,000.

FIG. 4. Film of the omentum of Rabbit R 2801, which had received two intraperitoneal injections of 20 mg. of leprosin in the form of a dry powder. The animal was killed 4 days after the second injection. The preparation was stained with neutral red and the photograph was taken while the cells were still living, and shows bands of fibroblasts. × 150.



Photographed by Louis Schmidt

(Sabin *et al.*: Waxes from *Mycobacteriuml eprae*)

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THE COLONY MORPHOLOGY OF TUBERCLE BACILLI

V. Influence of the pH of the Culture Medium on Colony Form*

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Plates 6 and 7

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Certain experiments recently reported have indicated that the pH of the medium on which tubercle bacilli are grown may have a profound influence on the colony morphology of these organisms. Moreover, it has been observed in this laboratory that the use of acid or alkali in isolating tubercle bacilli from infected tissue has a definite tendency to inhibit the development of certain colony variants without inhibiting the total amount of growth.

Steenken, Oatway, and Petroff (1) found that when human tubercle bacilli, Strain H-37, were grown on either Petroff's or Calmette's medium adjusted to pH 6.1, the colonies were characteristically granular and rough. By selection of colonies and repeated transplantation on these media, colony variants were obtained which possessed very low pathogenicity. However, when low, veil-like, spreading, stippled colonies were grown on Petroff's medium adjusted to pH 7.2, these variants possessed the full virulence of the parent undissociated strain. Similar observations were again reported by Steenken (2) who employed synthetic medium adjusted to pH 6.0 or pH 7.6. At pH 6.0 the growth of the human strain, H-37, was dense and compact, whereas at pH 7.6 it was veil-like and spreading. Both variants proved to be virulent for guinea pigs but animals inoculated with the variants grown at pH 7.6 died the more quickly.

Birkhaug (3) also studied certain relationships between the hydrogen ion concentration in the medium and the colony morphology of tubercle bacilli. He found maximal growth (dry weight) of S variants in acid medium, and of R variants in alkaline medium. He studied the reaction curve during growth of dissociated strains on fluid medium, observing that mammalian S forms and avian

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R forms render the medium more acid, while mammalian R forms and avian S forms cause increased alkalinity.

A short time ago we (4) observed that various strains of human tubercle bacilli in primary culture produced considerable numbers of smooth, rounded colonies, as well as flat, spreading, finely granular colonies, or others more coarsely granular and irregular in contour. In controlled experiments it was shown that greatest numbers of smooth colonies were obtained by growing the organisms on Corper's medium and by avoiding the usual procedure of destroying contaminating organisms with acid or alkali. The latter observation indicated that certain colony variants might be susceptible to acid or alkali, and therefore to differences in pH of the medium. A preliminary experiment (4) showed marked differences in colony form of organisms grown at various pH values, with a probable optimum pH for the smooth variants. The latter observation was confirmed and extended in experiments reported briefly elsewhere (5). The present communication is a detailed report of the cultural studies.

It will be shown in the following paragraphs that the colony topography in cultures of tubercle bacilli is profoundly influenced by variations in the pH of the basic medium used (Corper's). The variations to be described are not rigidly confined, but the more acid medium (pH 6.0) favors the growth of granular colonies, the more alkaline medium (pH 6.8 to 7.4) favors the growth of veil-like, spreading colony variants, whereas the greater number of smooth, glistening colonies develop on medium adjusted to pH values of 6.4 to 6.8. It will also be shown that these variations apply to strains of low virulence, as well as to strains of high virulence, and that the range of pH apparently optimum for development of smooth colonies varies with the type of organisms (human, bovine, or avian).

Materials and Methods

In order to apply the method to a study of strains of tubercle bacilli possessing widely varying pathogenic properties, twenty-five strains were used in the experiments. These included three avian, twelve bovine, and ten human type strains.[1] Data concerning the source and date of isolation of these strains are recorded in Table I. The pathogenic properties of nineteen of these strains were discussed previously (6), and for convenience are recorded in Table I.

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[1] In the preliminary report it was stated that eleven bovine and eleven human type strains were used. It has since been established (6) that one of the strains of human origin was of bovine type, which accounts for the discrepancy in these statements.

TABLE I

Strains of Tubercle Bacilli Used in Cultural Studies with Data Regarding the Isolation and Pathogenic Properties of Each

Strain of T. B.	Isolated by	Isolated from	Date of isolation	Relative virulence
Avian R	M. C. Kahn*	Fowl	About 1910	Low
" S	" " "	"	" 1910	" †
" TS	Theobald Smith	"	" 1930	High
Bovine B-1	E. R. Baldwin		1904	Low
" 32	Theobald Smith	Cow	Apr., 1928	"
" 33	" "	"	May, 1928	"
" 34	" "	"	Apr., 1929	Moderate
" 35	" "	"	" 1929	Low
" 36	" "	"	" 1929	High
" 37	" "	"	Oct., 1929	Low
" 38	" "	"	Mar., 1932	High
" 39	" "	"	Oct., 1932	"
" 40	" "	"	Jan., 1933	"
" Kilty	K. C. Smithburn	Human. Wrist fluid	Feb., 1934	Moderate
" Ravenel	M. P. Ravenel		About 1905	High
Human H-37	E. R. Baldwin	Human. Sputum	1905	Moderate
" Jamaica	J. Freund	Human. Tracheal node	Autumn, 1933	"
" MR	K. C. Smithburn	Human. Psoas abscess	Dec., 1933	"
" 3103	" " "	*M. rhesus.* Spleen	Jan., 1934	"
" 3104	" " "	Human. Knee biopsy	" 1934	"
" Thompson	" " "	Human. Psoas abscess	Feb., 1934	"
" Bell	R. M. Thomas	Human. Sputum	Reisolated Feb., 1934	"
" 3421	K. C. Smithburn	Human. Spinal fluid	May, 1934	"
" 3422	" " "	Human. Spinal fluid	" 1934	"
" Burroughs	" " "	Human. Sputum	June, 1934	"

* These R and S variants were supplied to us by Dr. M. C. Kahn, Cornell University Medical College, New York, on Sept. 18, 1930. They are the strains studied by Kahn (8) and Petroff (9, 10) in their respective researches on dissociation of avian tubercle bacilli.

† This variant, when obtained, was highly pathogenic but has since become attenuated without changing in colony form.

The basic medium used in the experiments was the glycerolated egg yolk medium with Congo red, proposed by Corper and Cohn (7). 3,600 cc. or 1,800 cc. lots of the medium were prepared and divided into eight or four flasks, each containing about 450 cc. The pH of each lot was determined by the glass electrode

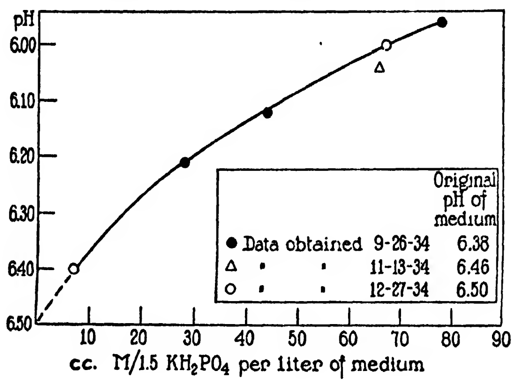

CHART 1

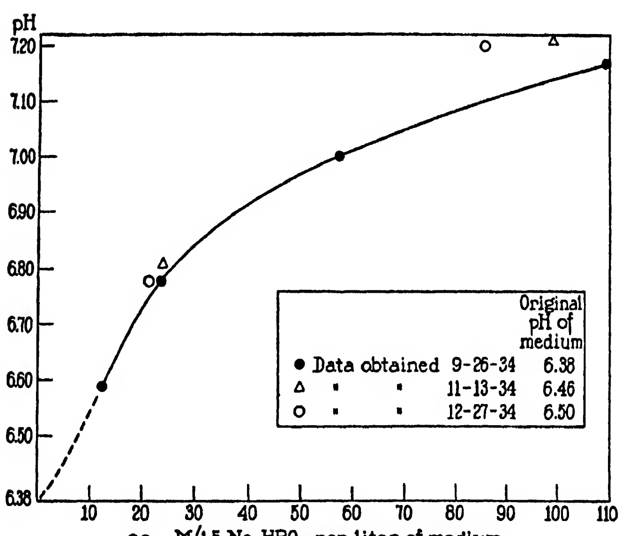

CHART 2

method.[2] The pH of each flask of medium was then adjusted to the desired value by fractional additions of M/1.5 KH_2PO_4 or Na_2HPO_4. No attempt was made to

[2] We wish to acknowledge the generosity of Drs. D. A. MacInnes, T. Shedlovsky, and Mary L. Miller, to whom we are indebted for all the pH determinations.

prepare the medium in such a manner that the pH would remain constant during growth; therefore only one buffer was added to any one lot, and this in just sufficient quantity to bring the pH to the desired value. This was a somewhat time-consuming procedure; but it was found that if a graph were plotted to show the amounts of buffer used to produce the various changes in pH, the curves obtained were quite smooth. Since various lots of standard medium varied in pH from 6.34 to 6.50, the data obtained in successive experiments did not all fall on the same curve (Charts 1 and 2), although the type of curve obtained was in each case similar.

Charts 1 and 2 are titration curves for Corper's medium against acid and alkaline buffers respectively. The data obtained in three experiments, wherein the original pH of the medium varied slightly, are superimposed on the charts. The

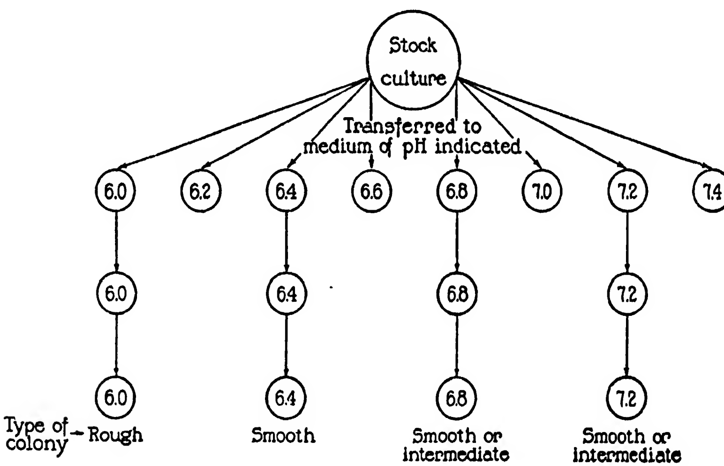

CHART 3

portions of the two curves represented as broken lines are so indicated because basal calculations were necessary in these regions and no absolute data were available. From these charts it may be seen that the addition of a definite quantity of either buffer, as indicated on the graphs, to medium of known original pH would give a final pH within 0.1 pH or less of a desired value. For absolute precision, titration curves for medium of various original pH values, with many additions of buffer, would be necessary. The methods employed, however, are wholly adequate for this type of work.

After the pH of each flask of medium had been adjusted, 5 to 7 cc. portions of the latter were placed in culture tubes of 20 to 25 cc. capacity, and the slants were inspissated in the usual manner. Sterility of the medium was determined by incubating at 37°C. for 2 to 4 days.

Since we desired only to determine the influence on dissociation of that environ-

mental change occasioned by variations in pH of the medium, no attempt was made to select colonies of different topography for transplanting. A quantity of organisms was merely removed from the parent culture with a platinum spatula and triturated just above the fluid level of saline in a small flask or tube; the container was shaken to suspend the organisms. Suspensions were prepared sufficiently dilute that colony topography could be ascertained. In order to avoid the possibility of eliminating variants which have a marked tendency to clump, the bacterial suspensions were not filtered. Transfers of each bacterial suspension were made in triplicate or duplicate by seeding each tube with 0.3 to 0.4 cc. of the suspension. In the first generation of cultures on medium of adjusted pH, all cultures of a given strain of organism were seeded from a single suspension of the stock culture. In subsequent generations transfers were also made from suspensions but always from medium of one pH to medium of the same pH value. Chart 3 illustrates this point and indicates certain general features of the colony topography observed at each pH.

Chart 3 shows the means by which subcultures of each strain of tubercle bacilli were derived in this study. The notations at the lower border indicate the pH values at which various colony variants appeared in greatest number.

After seeding, the cotton plug in each tube was covered with paraffin (m.p. 56–58°C.) and the tubes were incubated at 37°C. Each culture was twice examined with a binocular dissecting microscope during the 3rd and 4th or 5th weeks of incubation, and again after about 6 months. At each examination records were made pertaining to the amount of growth, the morphological variants present on each slant, and the approximate per cent of the whole which each comprised. The results to be discussed are those obtained in a study of three generations each, of twenty-five strains of organisms. In the first generation the medium was adjusted to eight different pH values so that there were approximately 600 cultures. Four values for pH of the medium were employed in the second and third generations, so that the whole number of cultures involved in this study was but slightly under 1,200. Contaminated cultures were discarded and not considered. They comprised less than 2 per cent of the total number of cultures.

RESULTS

Marked variation in colony topography of organisms grown at various pH values occurred in eighteen strains in the first generation. The remaining seven strains (all human type) showed similar changes in the second generation. Since the variations differed with the type of organisms, these will be discussed separately.

Avian Type Tubercle Bacilli.—At pH 6.0 and pH 6.2 a moderate number of typical rough colonies occurred in each generation, but these were most numerous in the first generation; and in each of the three generations the avian R variant (Kahn, see Table I) showed greater numbers of irregular, tortuous, granular

colonies than did the avian S or TS strains. However, even at pH 6.0 and 6.2 each strain of avian organisms showed many rounded, glistening colonies which were called smooth. Through the pH range 6.4 to 7.0 all the colonies of each of the avian strains were moist, shiny, rounded, glistening, and non-pigmented. The avian S and TS colonies were morphologically indistinguishable, although the former is avirulent and the latter highly virulent. At pH 7.2 (and pH 7.4, first generation) there was a tendency for the colonies to be flatter, but they were still regular in contour, glistening, and devoid of granular characteristics. Growth was slightly less vigorous above pH 7.0. After 6 months the smooth topography of the avian S and TS strains was maintained with little or no change; the avian R variant, however, showed secondary growth of "wormy," granular colonies, and some of the smooth colonies showed a narrow, spreading, veil-like border.

The avian strains at the various pH values showed differences in the ease with which they could be suspended in saline. The colonies grown at pH 6.4 and 6.8 were more easily suspended than those grown at pH 6.0 or pH 7.2. At pH 6.0 the organisms showed a moderate tendency to clump, while at pH 7.2 the growth was sometimes difficult to suspend, not because of a tendency to flocculate but because of a slimy stickiness which made dispersion difficult.

It is clear that smooth variants of avian tubercle bacilli, whether derived from virulent or avirulent strains, were produced over a broad range of pH; the rough variants appeared only on the more acid medium and even then not to the exclusion of smooth forms.

Bovine Type Tubercle Bacilli.—Each of the twelve bovine strains showed variations in topography dependent upon the pH of the medium in the first generation. At pH 6.0 and pH 6.2 the colonies were predominantly granular; some of them were characteristic, dry, tortuous, irregular, coarsely granular forms, rising sharply from the medium but adhering to its surface. These were designated rough. Other colonies, present in greater number, were more finely granular and had a tendency to spread over the surface of the medium; with prolonged incubation these became tortuous, "wormy," and matted together, and sometimes developed pale yellow pigmentation (especially bovine Strain 37). Small numbers of opaque, rounded colonies were present in the first weeks of incubation but became stippled or knobby in appearance later and often developed a granular veil. The latter type of colonies constituted about 11 per cent of the growth, whereas the granular variants constituted about 89 per cent. In the second and third generations the morphologic variants were similar but more moist in appearance until aged.

At pH 6.4 through pH 6.8 the granular variants of bovine strains showed a sharp decline in numbers, while the smooth, rounded, glistening colonies were most numerous. The latter constituted 23 to 90 per cent of all colonies in this pH range. They were usually quite moist and often semitransparent and somewhat jelly-like

in appearance. At these pH values the growths were composed almost exclusively of finely granular, spreading, veil-like colonies (intermediate) and glistening, rounded, non-granular forms (smooth). Smooth non-granular colonies of bovine tubercle bacilli grown at pH 6.4 and pH 6.8 are illustrated in Figs. 1 and 2 respectively. Fig. 2 may be compared with Figs. 5 and 9 of human tubercle bacilli grown at the same pH (6.8). These photographs illustrate the broader range for smooth colonies among bovine type organisms. With aging, the veil-like colonies became wrinkled but less "wormy" and matted than similar colonies grown on more acid medium; the smooth colonies, upon aging, often lost lustre and sometimes showed secondary growth of a veil-like border, or of superimposed colonies, but in general their topography was quite well preserved.

At the more alkaline pH values the bovine type bacilli produced almost exclusively a third type of colony which we have designated intermediate. These colonies were low, flat, spreading, semitransparent forms, usually with rounded margins and often with elevated centers. In some instances they were glistening and homogeneous; in others finely granular, but eventually most of them became finely granular. Truly rough colonies occurred but rarely and then only in the first generation. A moderate number of smooth appearing colonies were present in the early stages of growth but these eventually acquired the topography of the intermediate forms described above.

The bovine type bacilli then produced three variants instead of two, as in the case of avian organisms. The more granular forms occurred most abundantly on the more acid medium, the smooth forms in the mid-range of pH, and the third variant, designated intermediate, was produced almost to the exclusion of other forms on neutral or alkaline medium. Organisms grown at pH 6.0 were least readily suspensible, those grown at pH 7.2 somewhat more readily, while the organisms grown at pH 6.4 and pH 6.8 were in general much more readily suspensible.

Human Type Strains.—In the first generation of cultures grown on medium of various pH values, seven human strains showed only moderate differences in colony morphology, whereas three strains (including H-37) showed sharp variations depending on the pH of the medium. In the second and third generations all strains showed similar variations. The morphologic variations induced among the human type strains by altering the pH of the medium were in many respects like those noted among bovine type strains. Again three principal colony forms were observed. And again the more granular colonies were more numerous on the most acid medium, while the intermediate colonies were most numerous on the more alkaline medium. The apparent optimum range of pH for the development of smooth, glossy colonies was narrower than among bovine types however.

At pH 6.0 (and pH 6.2 in the first generation) granular, irregularly shaped colonies were more numerous than at other pH values. The number of these rough forms increased in successive generations from 2 to 8 and then 18 per cent of the whole number of colonies. At this pH, however, the predominating colonies were finely granular and somewhat veil-like, becoming wrinkled, tortuous, and "wormy" with aging and drying. The first generation of human strains showed no smooth colonies at pH 6.0, but in the second and third generations there were moderate numbers of glistening colonies, some of which became knobby or mulberry-like with age. Granular variants of human strains grown at pH 6.0 are illustrated in Figs. 3 and 7.

At pH 6.4 the smooth colonies were more numerous than at the other pH values, constituting from 25 to 47 per cent of the whole number of colonies. A very few rough colonies (1 to 5 per cent) were present, the remainder being of the variety designated intermediate. The smooth colonies of human strains were quite similar to the smooth variants of bovine organisms, but in general they were slightly less moist and slightly less regular in contour. Smooth variants of two human strains grown at pH 6.4 are illustrated in Figs. 4 and 8; they may be compared with smooth variants of a bovine strain in Figs. 1 and 2, and contrasted with granular variants of the same human strains grown on more acid medium (Figs. 3 and 7). The first generation of human strains grown at pH 6.6 showed fewer smooth colonies than the same cultures grown at pH 6.4, but more than were present at other pH values, indicating that the optimum pH may be slightly above pH 6.4. These smooth variants showed the same changes with age as the corresponding bovine variants: loss of lustre and growth of secondary colonies which were not smooth.

Through the pH range 6.8 to 7.2 (and 7.4 in the first generation) the predominating colonies closely resembled the bovine intermediate variants. They showed a marked tendency to spread over the surface of the medium. They were fairly regular in contour, often showed elevated centers, and were finely granular in appearance. In general these intermediate colonies of human tubercle bacilli were less glossy than the corresponding bovine variants. With aging and drying they frequently became wrinkled and tortuous. Moderate numbers of smooth colonies occurred at pH 6.8 and fewer at pH 7.2, especially in the early weeks of incubation, but these were far less numerous than at pH 6.4. Coarsely granular or rough variants were seldom seen either at pH 6.8 or 7.2, except after the culture was considerably aged.

The human type strains therefore produced variants similar to those of bovine tubercle bacilli. In the case of the bovine organisms, however, the optimum range of pH for smooth colonies on Corper's medium was pH 6.4 to 6.8, whereas among human strains large numbers of smooth colonies occurred only at pH 6.4. The pH range for intermediate variants of human tubercle bacilli was therefore

slightly more broad than in the case of the bovine organisms. Intermediate colonies of two human strains grown at pH 6.8 are shown in Figs. 5 and 9. Note the difference between these and colonies of bovine type bacilli grown at the same pH, Fig. 2. The intermediate colonies of the same human strains grown at pH 7.2 (Figs. 6 and 10) closely resemble those grown at pH 6.8.

DISCUSSION

Although it seems improbable that any one factor yet ascertained is wholly responsible for dissociation of tubercle bacilli, the results reported indicate the profound influence which a single factor, namely, pH of the culture medium, may have in determining the colony form of tubercle bacilli. That this is not the sole determining factor can be seen from the fact that pH control does not in any instance (except with avian bacilli) eliminate every variant except one. With the mammalian strains used, there were at least two variants present at each pH, although the results are striking enough when cultures grown at one pH value are compared with cultures of the same strain grown at a different pH value.[3] It must be stressed, however, that with prolonged incubation which allows for secondary growth, the influence of pH control becomes less evident. It is essential therefore in pursuing such a study that observations be made at least by the time cultures have been incubated for 1 month. Moreover, it is equally essential that some means of study be employed whereby colonies may be magnified four to ten diameters.

We have employed tubes as containers rather than plates because of greater assurance against contaminations and against drying of the medium; the latter is seemingly an important factor. For photographing cultures, however, tube containers are less satisfactory. In

[3] The recent work of Deskowitz and Shapiro (11) may bear upon, indeed, may offer an explanation for some of the facts under discussion. In a study of *Salmonella aertrycke* these workers found that S variants gave rise only to S variants, whereas R variants gave rise to both R and S daughter colonies. Under constant conditions a parent R colony gave rise to constant numbers of R and S daughter colonies. When environmental conditions were changed, the number of S colonies (derived from an R parent) was greatly increased, owing not to a deviation from the dissociation constant, but to the circumstance that the altered environment permitted a more rapid growth of the S cells.

order to overcome this difficulty, the entire slant may be removed from the tube with a sterile platinum spatula and placed in a sterile dish which is then covered with plate glass through which exposures are made.

The avian tubercle bacilli are seemingly less sensitive to alterations of pH than the mammalian strains. However, the avian and mammalian strains behave similarly in one respect, namely, the production of granular colonies on more acid medium. Although we have failed to observe flat, spreading, finely granular colonies of avian bacilli, these undoubtedly occur, as they have been observed by Petroff (10). It may be that the range of pH employed in our studies was not sufficiently broad (on the alkaline side) for the development of this variant. Smooth variants of avian strains of either high or low virulence are produced on Corper's medium over a wide range of pH.

Among mammalian strains, three principal variants occur. These we have designated rough, smooth, and intermediate. Rough forms of both bovine and human type bacilli occur in greatest numbers on the more acid medium used (pH 6.0). Smooth colony forms of human strains occur in greatest number at pH 6.4 and pH 6.6, whereas bovine smooth forms also occur in large numbers at pH 6.8. In both instances neutral or alkaline medium favors the intermediate variants.

The virulence of nineteen of the strains included in this study has been previously studied and reported (6). In the study of pathogenic properties, only the undissociated stock cultures of each strain were used. It was shown that among bovine type organisms the strains varied markedly in virulence from very low to very high, and that cultures of either high or low virulence, when recovered from inoculated animals, showed similar variations in colony topography; quantitatively, however, the more smooth colonies could be recovered from animals inoculated with the more virulent strains. Now in the present study variations in topography of colonies, occasioned by alterations of the pH of the culture medium, show that strains of low and of high virulence produce similar morphologic variants. If it could be established that none of the variants derived from avirulent strains possess enhanced pathogenic properties, then it could be concluded that dissociation is a general bacteriological phenomenon which occurs among non-pathogenic Mycobacteria, as well as among

pathogens. Such a conclusion would in no wise detract from a further statement that the major pathogenic properties of a virulent strain are associated with some one variant. Such studies have been made; the results, which confirm these views, will be discussed in a subsequent report.

It may be well to mention, however, that tests of virulence of some of the variants obtained in the present study have been made, and that they vary strikingly in pathogenic properties. The bovine rough variant derived from a highly virulent strain is only moderately pathogenic; the smooth variant is at least as virulent, if not more so, than the parent strain; and the variant designated intermediate on account of its colony form is truly intermediate in pathogenesis. None of the variants of an avirulent bovine strain showed enhanced pathogenic properties. Variants of the Burroughs human strain showed moderate differences in pathogenic properties. The pH 6.4 culture produced the most extensive lesions. The details of these experiments are mentioned here only to facilitate discussion.

These facts indicate that a delicate mechanism operates to determine both colony form and virulence of tubercle bacilli. If the factors of that mechanism are controlled so that maximum numbers of smooth colonies are produced, then greatest virulence is retained. The bacilli, when grown on more acid medium, produce fewer smooth and greater numbers of rough colonies and suffer attenuation. Sensitivity to alkaline medium is less, so that still another and but moderately attenuated colony variant is produced. It seems likely that this latter is the explanation for the fact that the pH range most suitable for the development of intermediate colonies does not occur between those values which are more suitable for R or S variants.

It cannot be assumed that the colonial variation due to pH would be identical were some culture medium other than glycerol-egg yolk medium used. It is possible that with some other culture medium the pH values optimum for certain colonial variants might be quite different from those observed in the present study.

The difference in optimum pH for development of smooth colonies which occurs between human and bovine tubercle bacilli might possibly be used as an additional means of typing new strains. In order to make the method effective, however, considerable experience in the

study of variants would be necessary; and one would need to study several cultures made on medium of each pH value.

It is interesting and perhaps noteworthy that the pH best suited to development of smooth colonies of mammalian tubercle bacilli is practically identical with that of the vacuoles in the cells which phagocytize the bacilli in the animal body (the vacuoles of monocytes react to neutral red showing a pH of about 6.5). This may indicate that at least one host factor is not unfavorable to the invading bacterium.

SUMMARY

The colony topography of tubercle bacilli is significantly affected by altering the pH of the culture medium on which the organisms are grown. Under the conditions of these experiments, avian tubercle bacilli produce two variants, rough and smooth. The former are most numerous on the most acid medium used (pH 6.0); the smooth colonies are obtained over a broad range of pH.

Three colonial variants of bovine and human tubercle bacilli are described. Both mammalian types produce greater numbers of rough colonies at pH 6.0. The bovine type strains produce greatest numbers of smooth colonies in the pH range 6.4 to 6.8, and intermediate colonies on alkaline medium. The human type strains produce greatest numbers of smooth colonies at pH 6.4 and large numbers of intermediate colonies at pH 6.8 and pH 7.2.

Included among the avian and bovine strains studied are organisms of widely varying pathogenic properties. Virulent and attenuated strains of a given type produce similar colonial variants under similar environmental conditions.

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EXPLANATION OF PLATES

Plate 6

Fig. 1. Smooth colonies of a bovine strain of tubercle bacilli, grown on Corper's medium at pH 6.4. Age of culture, 29 days. ×8.

Fig. 2. Smooth colonies of a bovine strain of tubercle bacilli grown on Corper's medium, pH 6.8. Age of culture, 29 days. ×8.

Fig. 3. Rough colonies of the Burroughs strain, human tubercle bacilli, grown on Corper's medium at pH 6.0. Age of culture, 36 days. ×8.

Fig. 4. Smooth colonies of the same strain as Fig. 3, grown on the same medium at pH 6.4. Age of culture, 36 days. ×8.

Fig. 5. Intermediate colonies, same strain as Figs. 3 and 4, grown at pH 6.8. Compare with Fig. 2, showing bovine tubercle bacilli cultivated under identical conditions. Age of culture, 36 days. ×8.

Fig. 6. Intermediate colonies, same strain as Figs. 3, 4, and 5, grown at pH 7.2. Age of culture, 36 days. ×8.

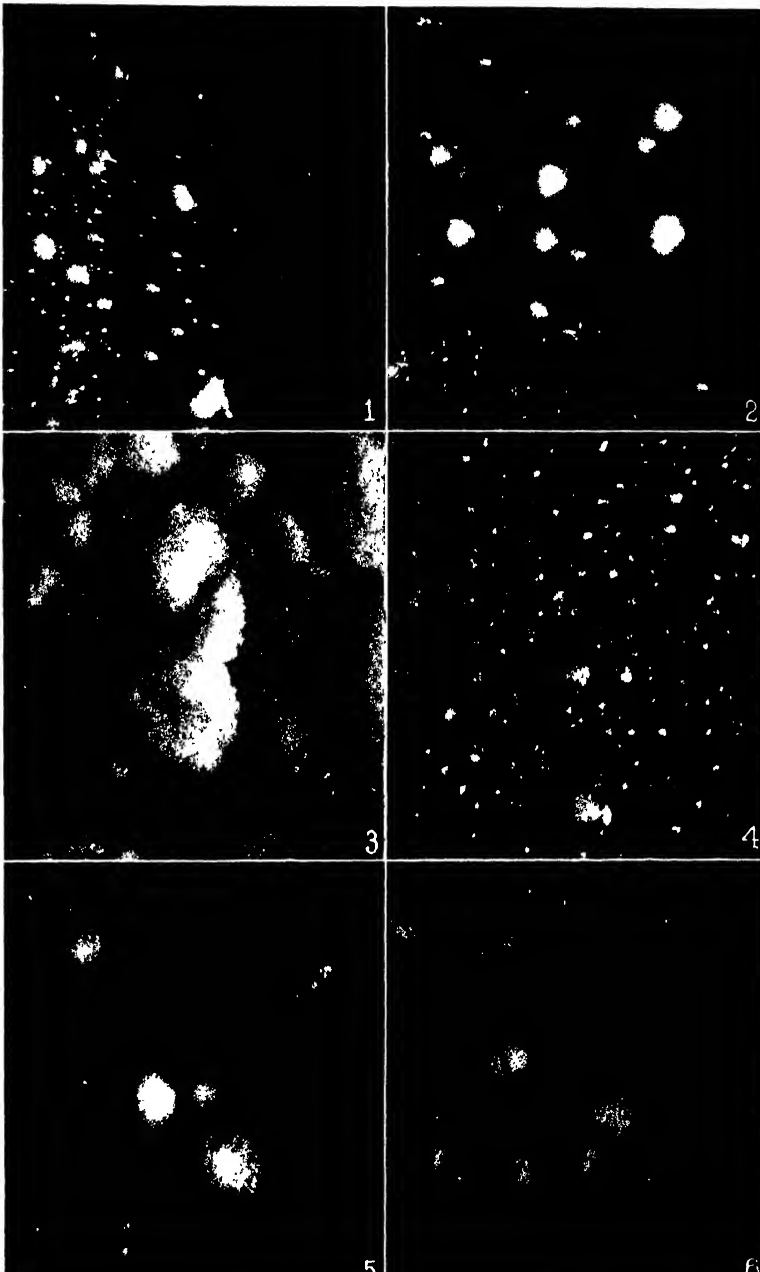
Plate 7

Fig. 7. Rough colonies of another human strain, Jamaica, grown on Corper's medium at pH 6.0. Age of culture, 105 days. ×8.5.

Fig. 8. Smooth colonies of the Jamaica strain, grown at pH 6.4. Age of culture, 105 days. ×8.5.

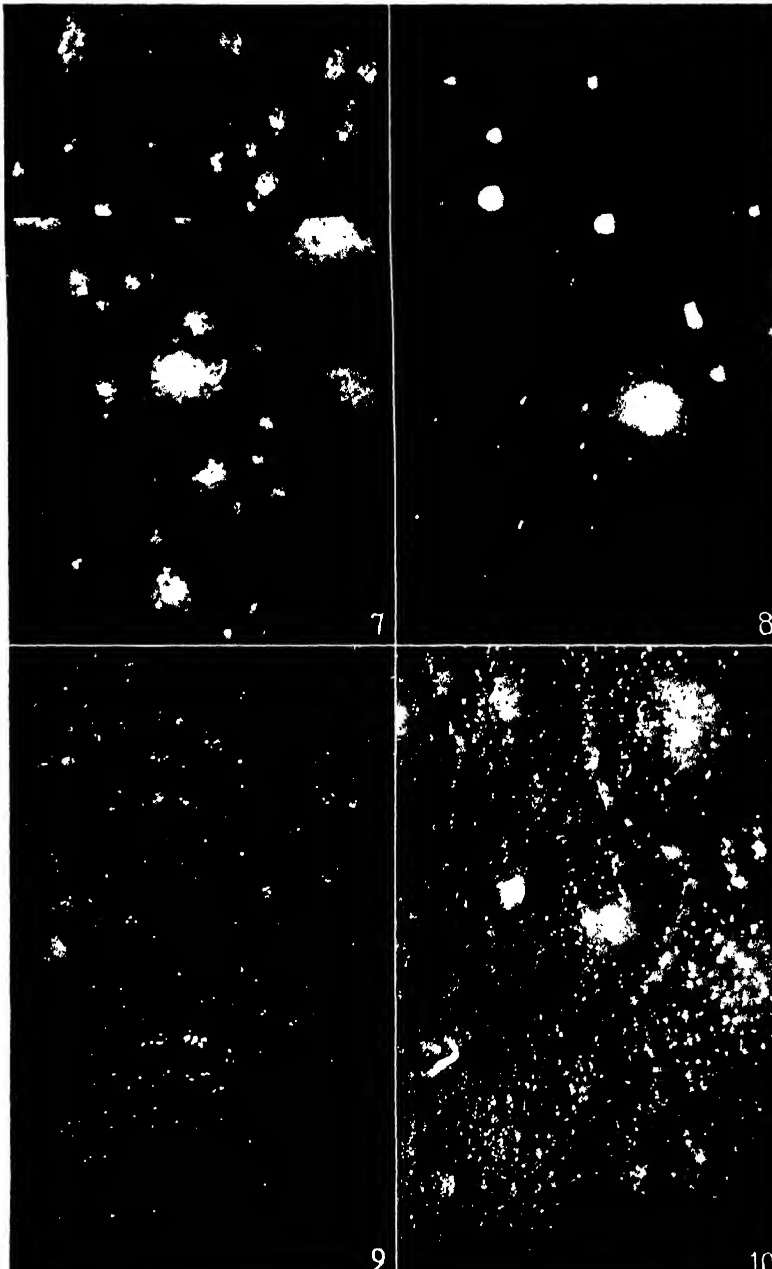
Fig. 9. Intermediate colonies of the Jamaica strain, grown at pH 6.8, also 105 days old. ×8.5.

Fig. 10. Intermediate colonies of the Jamaica strain, grown at pH 7.2. Age, 105 days. ×8.5.



Photographed by Louis Schmidt

(Smithburn: Colony morphology of tubercle bacilli. V)



Photographed by Louis Schmidt

(Smithburn: Colony morphology of tubercle bacilli. V)

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THE EFFECTS OF NASALLY INSTILLED VIRUS OF POLIOMYELITIS ON THE CEREBROSPINAL FLUID AND THE BLOOD OF MONKEYS

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While much has been written on the ultimate effects of neurotropic poliomyelitis virus dropped into the nose of *Macacus* monkeys and made to run over the olfactory nervous areas, little has been published on the immediate, remote influence of the presence of the virus on the nasal membranes.[1] The subject is one of real importance, if for no other reason than because of the commonly held belief that spontaneous mass immunization to poliomyelitis among children results from the chance presence in the upper respiratory tract of the widely disseminated virus, not only during the prevalence of epidemics, but even during interepidemic periods. That the nasal portal of entry of the virus is effective is shown by the frequency with which the paralytic disease arises among nasally instilled monkeys in the absence of

* I desire to acknowledge with many thanks the efficient help given me by Mr. Peter Haselbauer in carrying out the experiments on which the series of articles on poliomyelitis now in course of publication is based.

[1] Jungeblut and Hazen (1) subjected four *rhesus* monkeys to "extensive" spraying of the nose and throat with a 10 per cent suspension of poliomyelitis virus during a period of 2½ months. The spraying was done twice a week, each animal receiving 20 sprays of about 1 cc. Three animals survived the test period, and were bled 1 month after the last treatment. The serum was tested for antivirus, and the monkeys were inoculated cerebrally with virus. All three monkeys developed paralytic poliomyelitis, and the sera exerted no detected neutralizing action. The doses of virus (1 cc. of a 10 per cent suspension), and the virus (0.5 cc. of a 1 per cent suspension) and serum (0.5 cc.) mixtures, indicate either a strain of low virulence, or the use of overwhelming quantities in the tests. However, the results seem unequivocal, inasmuch as Flexner (2), employing more measured amounts of virus, also reported that monkeys refractory to nasal instillation of virus were devoid of serum antiviral activity and exhibited average susceptibility to the intracerebral injection of potent virus.

all demonstrable lesions in the infected membranes; and the pathogenic effects of the virus so inoculated are little, if at all, surpassed by its direct cerebral inoculation.

It is not possible to detect the immediate effect of virus introduced directly into the brain, because of the injury of the tissues attending the injection. It is, however, common knowledge that monkeys which resist such inoculations, remaining free from obvious, detectable symptoms of disease, are as receptive to a subsequent injection of more potent virus as are control animals. Indeed, monkeys may resist more than one cerebral inoculation and yet respond to a later injection of virus in a manner indistinguishable from that exhibited by previously uninoculated animals. Since the injection of virus, once or repeatedly, into or beneath the skin in monkeys induces active immunity, the failure of a corresponding state to develop in intracerebrally inoculated animals is worthy of emphasis.

While it is, as stated, impossible, because of associated effects, to determine the immediate action of the virus when introduced cerebrally, no such barrier exists to the detection of effects when the virus is brought into contact with the olfactory area in the nasal membranes. If, for example, the cerebrospinal fluid is withdrawn by cisterna puncture and the cells present in it be counted, the striking fact will emerge that the presence of the virus invariably produces changes in the cell content, irrespective of whether symptoms of disease do or do not appear (3).[2]

This delicately adjusted response of the cerebrospinal fluid to the presence of the virus of poliomyelitis on the nasal membranes has engaged our attention for some time. We have followed the phenomenon under a variety of conditions and circumstances, which it will be the purpose of this paper and succeeding ones to describe. In studying the reaction we have had in mind its relation, if any, to the immunity which may be induced in monkeys by means of successive inoculations of the virus; and collaterally, the immunity which arises in children who are exposed to the virus and respond to its presence with mild attacks of illness—in their nature poliomyelitic—or who, through an unperceived or undetected series of events, become, in the

[2] It should be stated at this point that the instillation of sterile salt solution into the nares of *Macacus rhesus* on 6 successive days does not affect the number of cells in the cerebrospinal fluid withdrawn by cisterna puncture.

common phrase, specifically spontaneously immunized and protected against such infection.

This first paper will deal with the manner in which normal monkeys respond to one or multiple instillations of virus with changes in the cerebrospinal fluid, in comparison with discoverable alterations, of an immunity character, in the animals treated.

Methods

Instillation.—1 cc. of a lightly centrifuged (300 revolutions per minute, for 1 minute) 10 per cent suspension of glycerolated monkey medulla and spinal cord, washed in two changes of salt solution, is instilled with a medicine dropper into each nostril of the animal.

For the second and for each subsequent nasal instillation, a new suspension is prepared, preferably from a glycerolated specimen other than that used previously. More consistent results can be obtained when suspensions are prepared from specimens which have been kept in glycerol less than 10 weeks.

The monkey is held by an assistant (no etherization is necessary for this procedure) in an upright position, with head bent backward. By attaching an ordinary rubber urethral tip to the dropper containing the virus, this device can be placed tightly against the nostrils and more force can be exerted, thus allowing the suspension to distribute itself to all parts of the upper nasal respiratory tract.

Cisterna Puncture.—The position of holding the monkey is important. After the animal has been thoroughly etherized, the back of the neck shaved and sterilized, an assistant places it on its abdomen, letting the head drop forward over the edge of the table; the assistant holds the head firmly with both hands.

A *sharp* hypodermic needle (1 inch cannula, No. 20 gauge) is inserted *vertically* almost its entire length until it reaches the cisterna magna region, which can be readily determined in the fingers by the sudden release of resistance. The stylet is then removed, and clear fluid is allowed to flow. The fluid is quickly taken off by a capillary pipette and placed in small Wassermann tubes.

Occasionally the first few drops of fluid may be tinged with blood, in which case pipetting is continued until a clear specimen is obtained.

Cells.—After gentle shaking, the cerebrospinal fluid is conveyed by a capillary pipette to the white cell counting chamber. No dye is added to the fluid. 4 square millimeters, or one large square in each corner of the usual 9 square millimeter cell counter, are counted each time.

If red cells are present, these can be eliminated by adding 3 per cent of an acetic acid solution, using equal parts of this solution and spinal fluid. A long pipette with a total capacity of 0.1 cc., divided into 100ths, is sufficiently accurate. The acetic fluid solution is drawn up to the 5th mark, after which the spinal fluid is brought up to the 10th or final mark. The entire contents are then expelled into a small, conical shaped test tube, allowing the mixture to remain in the tube for several minutes before counting.

Globulin Test.—The Noguchi butyric acid test brings out a positive reaction much earlier than do other methods.

(1) 0.5 cc. of a 10 per cent butyric acid solution, made up in salt solution, is added to 0.1 or 0.2 cc. of clear spinal fluid. The test tube is placed in boiling water and allowed to remain for 2 minutes in the boiling water bath.

(2) Then 0.1 cc. of N/10 sodium hydroxide is added and the tube is again placed in the boiling water for 1 or 2 minutes. Almost immediately, or in a few minutes, a precipitate forms and settles to the bottom in strongly positive reactions. If only a small amount of globulin is present, it may take up to an hour to form a precipitate.

Temperatures.—Rectal temperatures are taken daily, following the first instillation of virus and every day thereafter, usually in the morning, between 9:00 and 11:00 a.m. The thermometer is allowed to remain in the rectum for 1 minute. The Fahrenheit scale is used.

Protocols

TABLE I

Macacus rhesus Responding Symptomatically to One Course of Instillations. Temperatures, Cell Counts, Globulin, and Symptoms

No. of monkey	Virus strain, dosage, dates of instillation	Dates	Temperatures	Cells	Globulin	Symptoms
I	Philadelphia, 1932,			36 (normal)		
	4 doses, 3/27,	3/29	101.8	73		
	3/28, 4/5, 4/6/33	3/31	103	96	+	
		4/3	103	108	±	
	*Accelerating doses	4/5	102.6	193	±	
		4/6	102			
		4/7	104.6	158	+	
		4/8	104.6	166	+	Tremor, ataxia; right deltoid paralyzed
		4/10	104 4	290	+	No change
		4/11	105.4			" "
		4/12	103	188	+?	
		4/13	101	64	±?	More active. Recovered without residual paralysis

* The accelerating instillations affected temperatures, cell counts, and globulin content. They did not affect the paralytic symptoms.

TABLE I—*Concluded*

No. of monkey	Virus strain, dosage, dates of instillation	Dates	Temperatures	Cells	Globulin	Symptoms
II	Mixed, 2 doses, 6/14, 6/15/33			29 (normal)		
		6/16	102.2	65		
		6/19	104.2	165		
		6/20	104.6	320		Tremor
		6/21	104 6	405		
		6/22	103.6	570	+	Ataxia, followed by paralysis, arms and legs
III	Havana, 1934, 6 doses, 1/2–1/8/35			22 (normal)		
		1/9	103	36		
		1/11	104	148		
		1/14	105.6	496		Deltoid paralysis, followed by prostration
IV	Mixed, 2 doses, 1/8, 1/10/35			36 (normal)		
		1/12	103	140		
		1/14	105.2	504	+	Tremor, ataxia
		1/16	103.4	580		Arms paralyzed
		1/17				Prostrate
V	Mixed, 3 doses, 1/8, 1/10, 1/12/35		103.6	29 (normal)		
		1/14	103.6	490		Ataxia
		1/16	106	610	++	Arms paralyzed
		1/17				Prostrate
VI	Philadelphia, 1932, 2 doses, 1/24, 1/26/35			30 (normal)		
		1/26	101.2	35		
		1/28	105.2	374		
		1/30	103.2	580	+	Tremor, ataxia, arm paralysis
VII	Cooperstown, 1928, 3 doses, 2/18–2/23/35			27 (normal)		
		2/23	102.6	33		
		2/25	103.6	145		
		2/27	104.4	455		
	Accelerating dose	3/1	104.6	220		Tremor
		3/4	104	210		Deltoid paralysis
		3/6	103	365		
		3/8	104	390		Arms, legs paralyzed
		3/12	102.8	405		

TABLE II

Macacus rhesus Responding Asymptomatically, with Cell Changes. Temperatures, Cell Counts, Globulin, and Symptoms

No. of monkey	Virus strain, dosage, dates of instillation	Dates	Temperatures	Cells	Globulin	Symptoms
VIII	New York, 1933, 2 doses, 12/26, 12/27/33			48 (normal)		
		12/29	103.4	97		No symptoms
		12/31	103.2	120		
		1/2/34	104.4	410		
		1/5	102	640		
IX	Mixed, 1 dose, 2/13/34			42 (normal)		
		2/14	103.4	60		
		2/15	103	220		
		2/16	103	465		
		2/17	103.2	520		
		2/19	104.4	405	0	No symptoms
		2/20	104	260		
		2/21	102.4	125		
		2/24	102.8	102		
		3/5	101.8	44		
X	Mixed, 1 dose, 2/13/34			55 (normal)		
		2/14	103.4	60		
		2/15	102.2	110		(Sacrificed for histology)*
XI	Mixed, 1 dose, 2/13/34			35 (normal)		
		2/14	101 8	55		
		2/15	104	514	++	(Sacrificed for histology)
XII	Philadelphia, 1932, 2 doses, 2/5, 2/7/35			14 (normal)		
		2/7	103	45		
		2/9	103.2	614		No symptoms
		2/11	103.4	710		
		2/13	103.6	320		
		2/16	102.4	190		
XIII	Cooperstown, 1928, 4 doses, 2/18–2/23/35			27 (normal)		
		2/23	102.6	33		No symptoms
		2/25	103.6	145		
		2/27	104.4	155		
		3/1	104.6	220		

* Two *Macacus rhesus* were injected as follows: one cerebrally and peritoneally with a suspension of the mixed medulla, pons and cervical cord; the other in the same manner, with a suspension of the intervertebral ganglia. No symptoms resulted in either animal.

TABLE III

Macacus rhesus Given Two or More Courses of Instillations, the Earlier Nonsymptomatic, the Later Paralytic.
Temperatures, Cell Counts, Globulin, and Symptoms

No. of monkey	Virus strain, dosage, dates of instillation	Dates	Temperatures	Cells	Globulin	Symptoms
XIV	New York, 1933, 2 doses, 12/26, 12/27/33	12/26	102.6	48 (normal)		
		12/29	103.4	97		
		12/31	103.2	120		Fleeting symptoms, including excitement and partial ptosis
		1/2/34	104.4	410		
		1/5	102	640		
		1/8	101.4			
	New York, 1933, 6 doses, 2/5–2/10/34	2/7	103	105		
		2/9	103.4	62		
		2/13	102 6	100		No symptoms
		2/15	102.8	45		
		2/16	101.6			
*	Philadelphia, 1932, 3 doses, 3/7, 3/9, 3/13/35			26 (normal)		
		3/9	102.8	24		
		3/11	103	65		No symptoms
		3/13	101.8	34		
		3/15	103.6	64		
		3/18	101.6	43		
	Havana, 1934, 3 doses, 5/15, 5/17, 5/21/35			31 (normal)		
		5/18	103	33		
		5/21	103.2	37		
		5/22	105.6	265		
		5/24	106	580	+	Tremor, ataxia, legs paralyzed
		5/29				Prostrate
XV	New York, 1933, 2 doses, 12/26, 12/27/33	12/26	103	44 (normal)		
		12/29	103	58		
		12/31	102.6	230		No symptoms
		1/2/34	103.6	260		
		1/5	103	365		
		1/8	102.4			

* The 1933 New York and the 1932 Philadelphia strains acted alike; the 1934 Havana strain acted differently. In keeping is the neutralization of 0.2 cc. Philadelphia virus by 0.8 cc. of serum taken after the Philadelphia virus instillations.

TABLE III—*Concluded*

No. of monkey	Virus strain, dosage, dates of instillation	Dates	Temperatures	Cells	Globulin	Symptoms
XV—*cont'd*	New York, 1933,			50 (normal)		
	6 doses, 2/5–	2/7	102	64		
	2/10/34	2/9	102.4	125		No symptoms
		2/13	103.2	37		
		2/15	102	42		
		2/16	103			
	Havana, 1934, 3			24 (normal)		
	doses, 5/15, 5/17,	5/18	101.8	27		
	5/21/35	5/21	103.6	30		
		5/22	105.8	215		
		5/23	105.2			
		5/24	105.8	670		
		5/25	104.2			Tremor, ataxia, legs paralyzed
		5/28				Prostrate
XVI	New York, 1933, 5	1/2	103	65		No symptoms
	doses, 12/27/33–	1/4	103.2	80		
	1/2/34	1/6	103	130		
		1/8	103	335		
	New York, 1933,	2/5		67 (normal)		
	5 doses, 2/5–	2/7	103	205		
	2/10/34	2/9	102.8	535		
		2/13	105 2	620		
		2/15	103	212		
		2/17	104	420		Ataxia, weak arms
		2/19	104	450		Prostrate
XVII†	Mixed, 1 dose,	2/13		42 (normal)		
	2/13/34	2/14	103 4	60		
		2/15	103	220		No symptoms
		2/16	103	465		
		2/17	103.2	520		
		2/19	104.4	405		
		2/21	102 4	125		
		3/5	101.8	44		
	Mixed, 2 doses,	2/19		23 (normal)		
	2/19, 2/21/35	2/21	102.4	36		
		2/22	106			
		2/23	105.2	534	+	
		2/25	105.8			Tremor, ataxia
		2/26	104			Arm paralyzed
		2/27				Prostrate

† The same animal as recorded in Table II (No. IX). Bled Oct. 30, 1934, serum tested Nov. 3, 1934; 0.8 cc. serum did not neutralize 0.2 cc. Mixed Virus filtrate. The control monkey was prostrate on the 11th day.

TABLE IV

Macacus rhesus Resisting Multiple Instillations; Tested for Antivirus. Temperatures, Cell Counts, Globulin, and Symptoms

No. of monkey	Virus strain, dosage, dates of instillation	Dates	Temperatures	Cells	Globulin	Symptoms
XVIII	Mixed, 4 doses, March, 1933			No counts*		No symptoms
	Mixed, 4 doses, 1/10–1/13/34	1/13		65 (normal)		
		1/15	102.4	135		
		1/17	102.6	110		
		1/19	101.8	180		
		1/22	102	285		
		1/24	103	265		
		1/26	102.2	212		
		1/29	102.6	125†		
	Cooperstown, 1928, + New York, 1933, 6 doses, 1/29–2/3/34	1/29		125‡		
		1/31	101.8	105		
		2/2	102.2	320		No symptoms
		2/5	102.4	210		
		2/7	103	410		
		2/9	102	385		
		2/13	103	65		

* Bleeding of July 1, 1932, did not neutralize Mixed Virus in mixture of 0.7 cc. serum and 0.3 cc. virus filtrate.

† Neutralization conducted on June 19, 1935, of serum of Jan. 29, 1934, did not neutralize Mixed Virus in mixture of 0.8 cc. serum and 0.2 cc. filtrate.

‡ Cell count still high from previous instillations.

TABLE V

Macacus cynomolgus Instilled Repeatedly and Tested for Antivirus

Monkey	Course	Virus strain, dosage, dates of instillation	Symptoms
Cynomolgus A (Previously fed by stomach tube, without effect)	1st	Mixed, 6 doses, 11/2–11/7/31	6 days after the last instillation, deltoid became weak; no progression of paralysis. Complete recovery
	2nd	Mixed, 6 doses, 1/4–1/9/32	No symptoms
	3rd	Mixed, 3 doses (accelerating), 1/16–1/18/32	No symptoms
*Bled for serum, 3/10/32	4th	Mixed, 3 doses, 2/1–2/3/32	No symptoms
	5th	Mixed, 3 doses, 3/11–3/17/32	No symptoms

Neutralization Test

* Serum taken Mar. 10, 1932, after 4th course of instillation, mixed with virus in proportion of 0.9 cc. serum and 0.1 cc. filtrate of Mixed Virus; neutralized. Two injections given to 2 *rhesus* controls, Mar. 15 and 26, 1932 (acceleration); animals became paralyzed on 7th and 10th day respectively from same dose of virus filtrate.

Intracerebral Test

Apr. 22, 1932, 0.1 cc. Mixed Virus filtrate failed to induce symptoms. Two controls—one *cynomolgus* and one *rhesus*—became paralyzed on the 3rd and 8th day respectively from same dose of filtrate.

TABLE VI

Macacus cynomolgus Instilled Repeatedly and Tested for Antivirus

Monkey	Course	Virus strain, dosage, dates of instillation	Symptoms
Cynomolgus B	1st	Mixed, 6 doses, 11/2–11/7/31	No symptoms
	2nd	Mixed, 6 doses, 1/4–1/9/32	No symptoms
	3rd	Mixed, 3 doses (accelerating), 1/16–1/18/32	No symptoms
	4th	Mixed, 3 doses, 2/1–2/3/32	No symptoms
*Bled for serum, 3/10/32			
	5th	Mixed, 6 doses, 3/11–3/17/32	No symptoms

Neutralization Test

* Serum taken Mar. 10, 1932, after 4th course of instillations, mixed with Mixed Virus in proportion of 0.9 cc. serum and 0.1 cc. Mixed Virus filtrate, introduced into *Macacus rhesus*; no neutralization. Two injections of serum-virus mixture given: one, Mar. 15, 1932; the other (accelerating) on Mar. 26. Paralysis ensued on 10th day after the accelerating dose.

Intracerebral Test

Apr. 22, 1932, 0.1 cc. Mixed Virus filtrate injected into *Cynomolgus* B. Paralysis on 6th day. Two controls also became paralyzed.

TABLE VII

Macacus cynomolgus and Macacus rhesus Instilled Repeatedly and Tested for Antivirus

Monkey	Course	Virus strain, dosage, dates of instillation	Symptoms
Cynomolgus C	1st	Mixed, 6 doses, 11/2–11/7/31	No symptoms
(Mate to tube-fed *Cynomolgus* A)	2nd	Mixed, 6 doses, 1/4–1/9/32	Paralysis, 6th day
Rhesus D	1st	Mixed, 6 doses, 1/4–1/9/32	No symptoms
	2nd	Mixed, 3 doses (accelerating), 1/16–1/18/32	No symptoms
	3rd	Mixed, 3 doses, 2/1–2/3/32	No symptoms
*Bled for serum, 3/10/32			
	4th	Mixed, 3 doses, 3/11–3/17/32	No symptoms

Neutralization Test

* Mar. 15 and 26, 1932, (acceleration), 0.9 cc. serum and 0.1 cc. Mixed Virus filtrate into *Macacus rhesus*; no neutralization; paralysis 10th day after accelerating dose.

Intracerebral Test

Apr. 22, 1932, 0.1 cc. filtrate of Mixed Virus; paralysis 7th day.

In summing up the series of four monkeys (Tables V, VI, and VII) submitted to multiple courses of the instillation of virus, we find that, first, *Cynomolgus* A developed abortive symptoms of poliomyelitis from the first course, recovered, and proved durably immune, as shown by tests for antivirus and resistance to the cerebral injection of virus; second, that *Cynomolgus* B resisted five courses of instillation without producing detectable antivirus and without becoming in any degree refractory to a cerebral inoculation; and third, that *Rhesus* D behaved precisely as did *Cynomolgus* B, while *Cynomolgus* C resisted a first course of instillations merely to respond with paralysis to a second course given 2 months later.

Although these tests were carried out before the examination of the cerebrospinal fluid became a regular practice, there is every reason to believe that the succession of cell changes regularly occurred during the course of each series of instillations, as was always found to occur when examinations were made.

This series of tables completes the protocols of the main experiments. Tables VIII and IX which follow deal with complementary matters only. They are inserted to show: first, that the pleocytic cerebrospinal fluid contains no detectable virus at the height of the cell increases and even after acceleration inoculations; and, second, that repeated cisterna punctures in nonvirus-instilled animals do not change the average normal cell counts in the fluid.

TABLE VIII

Macacus rhesus Injected Cerebrally with Pleocytic Cerebrospinal Fluid from Nasally Instilled Monkeys

Monkeys	Virus strain, dosage, dates of instillation	Cells	Intracerebral inoculation	Symptoms
A	Mixed, 2 doses, 6/14, 6/15/33	29 (normal) 6/16–6/19, 65–165 Fluid withdrawn each day and pooled	6/20/33, 1.6 cc., 1st pooled fluid	No symptoms
		6/20–6/22/33, 320–570 Fluid withdrawn each day and pooled	6/28/33, 1.5 cc., 2nd pooled (accelerating) fluid	No symptoms
B	Philadelphia, 1932, 2 doses, 6/14, 6/15/33	19 (normal) 6/16–6/19/33, 37–118 Fluid withdrawn each day and pooled	6/20/33, 1.75 cc., 1st pooled fluid	No symptoms
		6/20–6/22/33, 470–515 Fluid withdrawn each day and pooled	6/28/33, 2 cc., 2nd pooled fluid	No symptoms

TABLE IX

Normal Macacus rhesus Controls, Cisterna Puncture, with Cell Counts

Monkeys	June 21, 1933	June 23, 1933	June 26, 1933	June 28, 1933	June 30, 1933
C	22	26	20	35	25
D	32	27	21	24	27

DISCUSSION

The protocols presented establish several important points. In the first place, they show unmistakably that the bringing of the virus into contact with the nasal membranes is never an indifferent process in monkeys. The response to the presence of active virus is prompt and invariable; and this response takes place irrespective of whether obvious clinical signs of disease do or do not arise, and always in advance of any such symptoms as may arise.

Most monkeys do not resist the initial course of instillations; the response has, perhaps, little relation to the number of instillations in monkeys of average susceptibility. Certain monkeys, however, while not markedly refractory, possess a degree of resistance above the average; they respond to the larger number of instillations, and there is inconclusive evidence that the spacing of the instillations makes a difference. Occasionally, monkeys in which the cells in the cerebrospinal fluid, while increased, remain at a low level, will develop higher cell counts if the animals are reinstilled on the 8th to the 10th day, a procedure to which in instances of intracerebral reinoculation we have given the name "acceleration." Instilled monkeys which pass through the usual course of incubation, showing beginning and then severe symptoms of poliomyelitis, have rising cell counts, coinciding with rising temperatures, while those which escape obvious clinical signs of infection tend to have lower cell counts and a correspondingly lower temperature range. Globulin as an index of inflammatory changes in the cerebrospinal fluid is irregularly demonstrable and bears an inconstant relation to high cell count.

Among the refractory monkeys which have resisted the initial course of instillations (although exhibiting changes in the cerebrospinal fluid) are some which, after a rest period, come down characteristically in response to a second course of instillations of the same virus as that employed in the first course. The reason for this disparity is not known. There remains a small residue of monkeys which, having resisted two such courses, now seems capable of resisting multiple courses of instillation without developing obvious symptoms of infection. None of these exceptionally refractory monkeys is indifferent to the virus instillations, for all react with cell changes in the cerebro-

spinal fluid. The refractory state, therefore, resides apparently in the nerve cells, the principal seat of usual virus attack—not in the nervous tissues as a whole.

Certain monkeys develop transient, slight, so called "abortive" symptoms of poliomyelitis as a result of the instillations. These animals have acquired increased resistance to virus injected into the brain, and their blood serum has become antiviral; and yet, reinstillation arouses cell changes in the cerebrospinal fluid qualitatively identical, quantitatively less marked, perhaps, than in monkeys failing to present obvious clinical signs of infection.

The series of reactions in the cerebrospinal fluid seems, therefore, independent of specific immune properties in the instilled monkeys. That there is no direct relationship between specific immunity and the changes induced in the cerebrospinal fluid is further shown by the important fact that, with present methods, active immunity invariably fails to develop in a detectable way, even in monkeys which have passed asymptomatically through multiple courses of instillation. These animals which are highly refractory to nasal virus (although always, probably, exhibiting cerebrospinal fluid alteration), never develop humoral antiviral properties, and are as susceptible to the cerebral injection of virus as are the normal controls.

The last observation brings us back to a consideration of the relationship which may exist between the monkeys nasally instilled which develop no symptoms of illness, and the many instances of general immunity arising spontaneously in human populations from the chance entrance of virus into the nasal passages. All that can be stated at present is that the two species behave in diametrically opposite ways. While there is indubitable evidence that unperceived immunization is taking place widely among human populations, there is also evidence that monkeys, while subject to the direct inoculation of poliomyelitis, are strongly refractory to the virus in highly dilute condition, such as occurs in ordinary contact exposures; and this refractory state is bound up with the complete inability of the monkey to initiate the requisite physiological changes which attend and lead to active immunity, independent of symptomatic response, when the virus is introduced in a way to reach the central nervous organs directly.

There is, therefore, a close correlation discernible between the unsuccessful cerebral and the asymptomatic nasal inoculation of virus. Probably the directly injected virus which does not lead to symptoms, produces changes corresponding to those readily detectable in the cerebrospinal fluid of nasally instilled animals; and as such asymptomatic, cerebrally injected monkeys have not been rendered immune, so those failing to respond with symptoms to nasal inoculation, similarly acquire no immunity. From this it would appear that in their fundamental physiological reactions to the virus of poliomyelitis, man and the monkey are widely divergent.

The virus passing by way of the olfactory area of the nasal membrane to the brain acts on tissues directly, and not through intermediation of the cerebrospinal fluid. This fluid remains constantly free of detectable virus. Even at the earlier stages (Table VIII) no virus can be demonstrated in the fluid; and in the course of active disease, when nerve and supporting tissue cells are severely injured, virus appears never to escape in ascertainable quantities into the fluid, either in man or the monkey. The virus displays strong avidity for cells, in this respect exceeding in cellular affinity other viruses which attack the tissue structures of the nervous system.

It would not be without interest to ascertain whether, in the process of unperceived mass immunization of children, cell changes occur in the cerebrospinal fluid. We know already that such changes attend mild illnesses believed to be poliomyelitis; opportunity to determine this point will arise in connection with outbreaks in institutions. And we may learn that during epidemic prevalences of poliomyelitis, cases which hitherto have been diagnosed mild poliomyelitis, merely because pleocytosis has been discovered in the cerebrospinal fluid of anxious and nervous individuals, may be the objective index of an otherwise unperceived process of active immunization taking place within them.

CONCLUSIONS

Macacus rhesus and *Macacus cynomolgus* exhibit a striking sensitivity to the presence of the virus of poliomyelitis on the nasal mucous membranes.

Irrespective of whether detectable symptoms of clinical poliomyelitis do or do not arise in the nasally instilled animals, the cere-

brospinal fluid changes quickly in response to virus placed in the nasal passages.

Two sets of changes occur in the cerebrospinal fluid: the constant and most pronounced change consists of increase in the content of white cells, chiefly of the lymphocytic type; the inconstant and less profound one consists of detectable amounts of globulin in the fluid (free from red corpuscles) withdrawn by cisterna puncture.

As early as 48 hours after instillation of the virus, a marked increase in cells is already detectable in the fluid; the increase grows from day to day, reaching a maximum sometimes in another day or two, sometimes not until 4, 5, or 6 more days elapse. In many instances a rise of temperature follows or coincides with the rising tide of cells; and the onset of clinical symptoms of disease bears also a relation to the cell count.

The number of cells in the cerebrospinal fluid and the temperatures tend to be higher in monkeys which develop paralytic symptoms; occasionally exceptions to this rule occur, in which instilled monkeys remaining asymptomatic exhibit high cell counts; very rarely do the latter show the higher temperatures.

Monkeys once instilled which fail to become symptomatically affected again react by cerebrospinal fluid changes to later courses of instillation. A second or still later course may induce paralysis; or highly exceptional or refractory animals may go through several courses of instillation without developing clinical symptoms, although never failing to respond with changes in the cerebrospinal fluid.

The virus instillations of *Macacus* monkeys do not lead to active immunization unless clinical symptoms of infection have resulted from the inoculations and attended the cerebrospinal fluid changes. In the complete absence of clinical symptoms the instilled animals fail to develop blood antiviral properties, and they are as susceptible to the cerebral injection of virus as are the control monkeys.

On the other hand, instilled monkeys which have shown even mild and fleeting (abortive) clinical symptoms of infection, resist cerebral inoculation and exhibit blood antiviral or neutralizing properties.

Monkeys which have developed clinical symptoms of disease and have recovered are, as stated, actively immunized; they remain, however, sensitive to the presence of virus on the nasal membrane, reacting

with cerebrospinal fluid changes, differing only in degree from the nonimmune animals.

Detectable virus does not appear in the pleocytic cerebrospinal fluid at any stage of the pathological processes.

The current belief is that mass immunization is proceeding in an unperceived manner through the chance entrance of virus into the nasal passages of children. It is not known whether, apart from all symptoms of disease, cerebrospinal fluid changes occur in the course of this unexpressed, spontaneous process. A large gap seems to exist between man and the monkey in the capacity of the former to become immunized by way of the nasal membrane, and the inability of the latter to do so. It is common knowledge that monkeys do not become immune through unsuccessful cerebral inoculations of virus, and the same seems to be true of the nasal channel of virus penetration into the central nervous organs.

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EXPERIMENTAL STUDIES ON ENCEPHALITIS

IV. Specific Inactivation of Virus by Sera from Persons Exposed to Encephalitis, St. Louis Type, 1933

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With a Note on the Evaluation of the Results of Mouse Tests of Sera

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Experiments on the probable virus nature of the encephalitis prevalent in St. Louis during the summer of 1933 have been reported by Muckenfuss, Armstrong, and McCordock (1), and by Webster and Fite (2, 3). The former workers obtained a virus by inoculating brain tissue from fatal cases into *Macacus rhesus* monkeys; the latter recovered a similar virus by inoculating the brain tissue into special mice. A further step in establishing this virus as the specific cause of the epidemic was the demonstration by Webster and Fite of the inactivation of the virus by human convalescent sera (4). This finding, confirmed by Wooley and Armstrong (5), and Muckenfuss (6), will now be described in detail.

Technique

The effect of serum on the virus was studied by means of the familiar protection test. Virus in suitable dilution was mixed with equal volumes of undiluted serum and injected intracerebrally into mice. The survival time of the injected animals was taken as a measure of the protective action of the sera.

Strain 3 virus (2, 3) was used in all experiments since it proved similar to other strains in every respect tested. Brains from one or two mice prostrate 3 to 5 days following an intracerebral injection of mouse brain virus, were triturated with alundum, diluted one part by weight of brain virus to 50 parts by volume of hormone broth, pH 8.0, centrifuged 10 minutes at 1,000 r.p.m., and the supernatant made up further in serial tenfold dilutions with hormone broth. 0.3 cc. of each chosen dilution of the brain virus was mixed thoroughly with 0.3 cc. of serum

in a test tube. The preparations were incubated 2 hours at 37°C., left at room temperature 2 hours, and then each taken up in a 0.25 cc. tuberculin syringe with 0.25 inch 26 gauge needle and injected intracerebrally in 0.03 cc. amounts into four to six mice lightly anesthetized. Precautions for asepsis were observed throughout. The condition and survival time of the injected mice were recorded for 21 days.

The choice of mice proved a significant factor in maintaining a maximum and similar degree of infectivity of the virus and in reducing irregularities in survival time of mice within and between tests. The unselected Rockefeller Institute stock mice (2) maintained the intracerebral infectivity of the virus at varying levels between 3×10^{-6} and 3×10^{-8} gm. of infected mouse brain and gave irregular survival times. Selected Rockefeller Institute resistant (virus-susceptible mice) (2) and selected Swiss mice proved the animals of choice, since they maintained the infectivity of the virus at about 3×10^{-9} gm. of infected mouse brain and proved most uniform in their response to the virus. All mice were free of intercurrent infection and came from our own breeding stocks. They were 4 to 6 weeks old and weighed 18 to 22 gm. When, on rare occasions, a doubt arose as to whether an injected animal died of encephalitis, brain sections were taken and tissue passed to another animal to establish the diagnosis. To test the uniformity of the mice, four to six were injected with each virus-serum mixture. To test the infectivity of the virus in each experiment, virus plus non-contact serum mixtures were injected into mice in dilutions of 10^{-3} to 10^{-7} inclusive.

Tests were made as soon as possible after withdrawal of blood, since protective bodies against this virus were found to decrease in quantity with *in vitro* age of serum (3). Protective sera diluted beyond 1 to 10 failed to react; hence tests were made at a final serum concentration of 1 to 2. Uncontrolled variations between experiments were checked by testing a greater part of the unknown sera at least twice, the non-contact control sera three to nine times, the doubtful sera two to four times, and some of the protecting sera two to eight times.

The test as now standardized is made with one non-contact and one known protecting serum as controls, together with five to fifteen test sera. The control sera are prepared to give virus dilutions of 10^{-5} to 10^{-7} inclusive, and the test sera to give virus dilutions of 10^{-5} and 10^{-6}. Each dilution of virus-serum mixture is injected intracerebrally into four Swiss mice.

Standardization of Protection Test

The first few protection tests were devoted primarily to standardization of the technique including the proper manipulation and dilution of the virus, determinations of stability of infectivity of the virus and of protective activity of a given serum, variations in effect of different normal non-contact sera, and variability of results between tests. The protocol of Test 3, for example, published elsewhere (7), besides

demonstrating the protective effect of St. Louis convalescent sera, is an instance in which the titre of the virus was limited to the 10^{-4} dilution by the technique employed. Following these early tests, the titre of virus has remained uniform at the 10^{-7} dilution. Again, it was learned that the protective titre of the human convalescent sera decreased with *in vitro* aging in the same manner as in the case of hyperimmune monkey sera (3).

While this standardization of procedure was being accomplished, it appeared that certain sera protected the injected mice fully against 100 killing doses of virus, but that others protected to a considerably less degree. What, then, was to be the basis of evaluating the protective effect of unknown sera?

The problem, a statistical one, was studied by Dr. H. Muench, of the International Health Division of the Rockefeller Foundation, and the results are given below. The data comprised two to nine titrations on each of thirteen normal non-contact sera, and one to six titrations on each of 267 unknown sera. Of these, 184 were classed later as non-protective and 67 as protective.

NOTE ON EVALUATION OF RESULTS OF MOUSE TESTS OF ENCEPHALITIS SERA

In attempting to establish criteria for distinguishing positive (protecting) from negative (non-protecting) sera, the first procedure was to find out what happens to "unprotected" mice that receive virus. For this purpose there was available a set of test results on a series of known normal, non-contact sera which had been used repeatedly as controls for test runs.

It became apparent that conditions in the first six test runs were widely divergent from those in the seventh and following. The latter group was very uniform. For this reason all findings have been based entirely on the analysis of tests after the sixth run. The results in the normal non-contact serum group are summarized in Table I.

Mortality rates are not well determined by one or two deaths even in a group of about 150, so that the rates at 10^{-4} and 10^{-5} cannot be regarded as very definite. The mortality at 10^{-6} is based on only forty-seven mice and therefore it is quite unreliable. The factors most certain, at least for the first two dilutions, are the average time of death after inoculation and its standard deviation.

The tests were done as routine on groups of four or of six mice. Now the standard deviation of the mean time of death in a sample of six mice at 10^{-4} would be $0.7810/\sqrt{-}$ or 0.3188 days from the mean value of the total. In other words, a mean time of death of 6.01 days would be twice the standard deviation

above the expected and this would occur accidentally in unprotected mice only some twenty-three times in 1000. Likewise, a mean time of death of 6.33 days would exceed the expected by three times the standard deviation; this could be accidental only thirteen times in 10,000.

For 10^{-5} dilutions and for four-mouse groups, the appropriate values are used. In this way it is possible to arrive at the criteria given in Table II. Here the + value corresponds to twice the standard deviation above the average; ++ is three times. Values as large as the latter or larger are almost certainly not due to chance and the corresponding sera cannot be called "negative."

This provides a criterion of what is not a negative serum. The question of what cannot be positive is still to be answered: it is not known whether positive

TABLE I

Virus dilution	No. of mice	No. dying	Mortality rate	Day of death	
				Average (mean)	Standard deviation
10^{-4}	148	147	0.9932	5.3537	0.7810
10^{-5}	146	144	0 9863	5 9514	0.9953
10^{-6}	47	37	0.7872	6.7027	0 8339

TABLE II

Average Survival Time in Days

Dilution	4 mice		6 mice	
	+	++	+	++
10^{-4}	6.13	6.53	6.01	6.33
10^{-5}	6.95	7.44	6.76	7.17

sera behave so differently that the average time of death is necessarily longer than in the case of negatives.

This cannot be answered from a study of known negative sera. It would be difficult to establish the distribution of longevity among "protected" mice from the results of tests of unknown sera, since these are evidently a mixture of positive and negative. A criterion based on mortality rates might be more definite if mortalities in protected and unprotected mice are sufficiently distinct and can be closely evaluated. In addition, such a criterion would be simpler and easier to apply than one based on length of life.

The problem then is to evaluate the two different mortalities at different virus dilutions and to find the point at which there will be the sharpest difference between them. Here there should be the least overlapping of criteria with consequent throwing of results into an "inconclusive" group.

In essence this is a study of binomial distributions. A group of tests, each test comprising the same number of mice among which there is a constant mortality rate would, in the long run, be distributed in a perfectly stable pattern. For example, 1000 groups of six mice each, with a mouse mortality of 0.9, would have the following most probable distribution:

6 deaths	532
5 "	354
4 "	98
3 "	15
2 "	1

while if the mortality were 0.2 we should expect:

5 deaths	2
4 "	15
3 "	82
2 "	246
1 death	393
0 deaths	262

As a starting point, we may assume that the distribution of test results (by number of mice dying) of any group of unknown sera is made up of a mixture of two such single distributions. One would be the scatter produced by a high mortality in unprotected mice which receive normal serum, which would have a peak at high numbers of deaths. The other, composed of test results in protected mice, would have a peak at a lower mortality. There are three unknown factors to be found: (*a*) the mortality rate of the unprotected group (= negative sera); (*b*) the mortality of the other group (protected mice or positive sera); (*c*) the proportion of each which makes up the entire number of tests; *i.e.*, the number of positive and of negative sera included.

These three factors may be derived from any actual array of test results mathematically in a perfectly straightforward way. Due to sampling variations, the actual distribution of a series of tests would hardly ever be exactly the expected one. For this reason the values we get for the factors are the best values, meaning those which will give the closest approximation to the series we are dealing with since we can hardly expect absolute concurrence.

In Table III is given, as an illustration, an actual distribution; in this case that of 110 tests, using four mice each, at a virus dilution of 10^{-5}. A survivor is a mouse alive on the 21st day after inoculation, when it was dropped from observation. Just below the actual number of tests in each mortality group is the hypothetical number calculated on the basis of the three factors obtained from the actual distribution. It will be seen that the correspondence is very close; the main difference is that there are actually rather more two-death results than might be expected.

If the calculated mortality rates are accurate, it would be expected that any other group of tests at the same dilution would give comparable results. The

third factor (distribution of positives and negatives) would vary from group to group depending on how many positive sera happened to be included in each

As a matter of fact, the unprotected mortality is very constant. This is illustrated in Table IV which summarizes the values obtained by analyzing different sets of data. Four-mouse and six-mouse groups must be separated for study on a binomial basis. The two different strains of mice were also kept separate in

TABLE III

Results of Four-Mouse Tests at 10^{-5} (Swiss Mice)

No. of mice dying	4	3	2	1	0	Total
No. of tests (actual)	67.0	10.0	7 0	9.0	17.0	110.0
" " " (calculated)	66.5	12 2	3.7	11.1	16.5	110.0

Calculated rates on basis of: 0.9571 mortality in 79 negative tests.
0.1455 " " 31 positive "

TABLE IV

Mortality Rates Derived from Test Results on "Unknown" Sera

Dilution	Mouse strain	No. of tests	Calculated mortality rates		"Unprotected" rates from normal sera (Table I)
			"Protected"	"Unprotected"	
10^{-4}: 6 mice	VS	33	0 6504	1.0	
	Swiss	95	0.4485	0.9943	0.9932
	VS	27	0.1382	0 9841	
4 mice	Swiss	99	0.6954	1.0	
10^{-5}: 6 mice	VS	43	0.1712	0.9717	
	Swiss	92	0 2800	0.9760	0 9863
	VS	27	0.0639	0.9463	
4 mice	Swiss	110	0.1455	0.9571	
10^{-6}: 4 mice	VS	27	0.0739	0.9186	0.7872
	Swiss	67	0.1076	0.9153	

case there should be a difference in their reactions. Known normal sera were excluded.

Not only are the unprotected mortalities homogeneous within a given dilution; they agree quite well with the mortality rates in known normal sera as given in Table I. The average values of 0.9640 at virus dilutions of 10^{-5} and 0.9160 at 10^{-6} are probably very close to the true mortalities. This permits the statement that, in negative sera, the expected occurrence of surviving mice would be as given in Table V. Unprotected mortality appears to have a fixed value and the

occurrence of five or six survivors in a test group of six mice, for instance, would throw a serum out of all reasonable probability of being negative.

The answer to the search for a protected mortality is nothing like as clear. It appears that there is no such entity, but rather a band of mortalities covering a considerable range which may reflect variations in protective power in different sera. Such spreading is indicated by the wide fluctuations between the calculated values for different groups in the same dilution (Table IV). The number of positive tests in each group is comparatively small and the variations show the effects of sampling from a widely spreading field. Confirmation of this conception of protected mortality as a band instead of a point is found in the quite regular excess of actual over computed results around the 50 per cent mortality point, which is shown in Table III.

But the computed protected mortality must be a "centering constant" of some sort. That is, it must be somewhere around the middle of the band of mortalities, some values being higher and some lower. In that case the lower the computed

TABLE V

Expected Survivors with Negative Sera

Dilution	2 or more	3 or more	4 or more
10^{-5}: 6 mice	176	8	— per 10,000 tests
4 "	74	2	— " 10,000 "
10^{-6}: 6 "	842	97	6 " 10,000 "
4 "	378	23	1 " 10,000 "

value, the less spread is to be expected. If the computed mortality is 0.5, the scatter of actual values might conceivably be quite even from 0 to 1.0. But if its value is 0.1, then it cannot be an average of a series which runs heavily to high values.

From this viewpoint it is possible to discuss results at different virus dilutions and their bearing on setting up criteria.

Dilution 10^{-4}. Negative sera produce scarcely any survivors, but the mortality among protected mice is so high that many positive sera, especially if weakly protective, will show few or no survivors and so cannot be differentiated.

Dilution 10^{-5}. The mortality in negative sera is still high, so that a test running to three or more survivors is quite certainly positive (Table V). Protected mortalities are evidently at a lower level than at 10^{-4}, but it might be expected that a weakly protective serum would show a sufficient number of deaths to throw the results into the possibly negative category.

Dilution 10^{-6}. Although lower, unprotected mortality is still sufficiently high to make the occurrence of three or more survivors indicative of a positive

serum. Protected mortality now centers about a value around 0.1 or less and in all probability seldom is high enough to confuse protective with non-protective sera.

Results at virus dilutions of 10^{-6} are thus undoubtedly the most sensitive and delicate. Dilutions were not carried farther than this: at 10^{-7} protected mortality would be lower but so would unprotected, and there might be less certainty in differentiating between the two than at 10^{-6}. It is impractical to embody dilutions of 10^{-4} in the criterion as the two mortality rates are so nearly alike.

As finally set up, the criterion of positive protection on the basis of mortality is as follows:

TABLE VI

Criterion for Test Results

No. of survivors*	0	1	2	3	4	5	6
10^{-5}: 6 mice	−	−	±	+	+	+	+
4 "	−	−	+	+	+		
10^{-6}: 6 "	−	−	−	+	+	+	+
4 "	−	−	±	+	+		

* At 21 days.

A number of sera, by this criterion, are positive at 10^{-6} but negative at 10^{-5}. This would be expected in the case of weakly protective sera according to the interpretation of the analysis. The reverse result should be found very seldom and it has not, in fact, occurred so far. Confirmation of the conception of such positive-negative results as weak positives is seen in the fact that the same sera are almost invariably outside the range of possible negatives on the basis of average length of life (Table II).

A ± result (Table VI) must be regarded as inconclusive unless the result at another dilution is clearly positive.

The final criterion for use in practise may be established as follows:

+ at 10^{-5} and + at 10^{-6}: ++ (strongly positive)
$\left.\begin{array}{l}- \text{ at } 10^{-5} \text{ and } + \text{ at } 10^{-6} \\ \pm \text{ at } 10^{-5} \text{ and } + \text{ at } 10^{-6}\end{array}\right\}$: + (weakly positive)
− at 10^{-5} and ± at 10^{-6}: ± (inconclusive, probably negative)
− at 10^{-5} and − at 10^{-6}: − (negative, no protection)

where the +, ±, and − values are determined from the number of survivors as given in Table VI.

Results with Sera from Persons with No History of Exposure to St. Louis Encephalitis

Sera were collected from September, 1933, to date from healthy non-contacts and from cases of encephalitis and poliomyelitis mostly in

hospitals in eastern and north central states.[1] The cases chosen were believed to have received careful clinical study, a matter of prime importance in determining the specificity of the reaction between serum and virus. The sera from the healthy non-contacts described below, when mixed with virus in dilutions of 10^{-4} to 10^{-6} and injected into mice, gave mortality rates of 99 per cent, 98 per cent, and 78 per cent, respectively, and average survival times in days of 5.3, 5.9, and 6.7 (Table I). The other sera, under similar conditions, gave mortality rates of 99 per cent, 96 per cent, and 91 per cent (Table IV), indicating that practically all mice given these sera plus virus in dilutions as low as 10^{-6} die in 5 to 7 days.

TABLE VII

Criteria for Protective Serum

Dilution of virus	Average survival time		No. of survivors	
	4 mice tests	6 mice tests	4 mice tests	6 mice tests
	days	*days*		
10^{-4}	6.53	6.33	—	—
10^{-5}	$\boxed{7.44}$	7.17	2	3
10^{-6}	—	—	3	3

Healthy Non-Contacts.—Sera from thirteen adults working in medical institutions in or near New York have been tested one to eight times with negative results (Table VII). Protocols of repeated tests with three of these sera are given in Table VIII, showing the uniformity of results subsequent to Test 14. In

[1] The authors are grateful for the generous cooperation of physicians and hospital authorities in calling our attention to cases of encephalitis, collecting and sending sera, and supplying clinical data. Mentioning all these collaborators by name is regarded as impractical. Most of the sera from the cases of chronic encephalitis with Parkinson sequelae were sent by Dr. David Marine, Montefiore Hospital, New York; Dr. C. H. Andrewes, National Institute for Medical Research, London; Dr. E. A. Carmichael, National Hospital Queen Square, London; Drs. Josephine B. Neal and Frederick Tilney, Neurological Institute, New York; and Dr. M. W. Raynor, Bloomingdale Hospital, White Plains, N. Y. Professors R. Inada and K. Kakinuma sent sera from twelve cases of Japanese B encephalitis, and Professor I. Takaki sent sera from three similar cases plus samples of hyperimmune sera and virus A and B in glycerine. Dr. G. F. Kempf forwarded sera from cases of meningoencephalopathy in Indianapolis.

TABLE VIII

Repeated Tests with Three Sera from Healthy Non-Contacts

Serum	Test	Date	Virus-serum dilution				
			10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
		1933					
C	13	Nov. 23	4, 5, 6, 7	5, 5, 5, 6	5, 6, 6, S		
	14	" 29	4, 4, 4, 6	4, 5, 6, 7	6, 6, 9, S		
	16	Dec. 20		4, 5, 5, 5, 6, 6	5, 5, 5, 6, *, *		
		1934					
	28	Apr. 25		5, 5, 5, 6, 6, 6	6, 6, 6, 6, 7, 7		
	43	Dec. 4		6, 6, 6, 6	6, 6, 6, 7	7, 7, 7, 8	8, S, S, S
		1935					
	45	Jan. 18		5, 5, 5, 6	6, 6, 6, 7	6, 6, 7, 8	6, 6, 11, 12
	47	Mar. 7		5, 5, 6, 6	6, 6, 6, 6	5, 7, 7, 7	8, 8, S, S
	48	" 12		5, 5, 5, 5	5, 5, 5, 6, 6, 7	6, 6, 7, 7, 7, 9	7, 8, 8, 9
	49	May 8		6, 6, 6, 6	5, 6, 6, 7	6, 6, 7, 7	7, 8, S, S
	50	" 24		5, 5, 6, 6	6, 6, 7, 7	7, 7, 9, 9	8, 10, S, S
	51	June 5		5, 6, 6, 6	5, 6, 7, 7	5, 6, 6, 7	6, 6, 7, 7
	52	" 10		*, 5, 5, 6	5, 5, 6, 6	5, 6, 6, 7	6, 7, 8, 8
		1934					
J	28	Apr. 25		5, 6, 6, 6, 6, 6	6, 6, 6, 8, 8, 8		
	36	Oct. 10		5, 5, 5, 6, 6, *	5, 5, 5, 6, 6, 6		
	39	" 25		4, 5, 5, 5	4, 5, 5, 7	6, 6, 7, 7	
		" 30		4, 6, 6, 8	6, 6, 6, 6		
		1935					
	46	Jan. 31		5, 6, 6, 6	5, 6, 6, 6	6, 7, 8, 8	6, 8, 9, S
		1933					
H	12	Nov. 16	4, 5, 5, 6	5, 5, 6, 6	5, 6, 6, 6	5, 5, 7, 9	
		1934					
	28	Apr. 25		6, 6, 6, 6, 6, 7	6, 6, 6, 6, 7, 7		
	33	Sept. 25		5, 5, 5, 5, 5, 5	5, 5, 5, 6, 6, 9		
	41	Nov. 12		5, 5, 5, 5	5, 5, 7, 7	7, 7, 7, 7	7, 7, 7, 7

S = mouse remained well 21 days.
Blank spaces indicate dilution not tested.
* = mouse died from trauma.

contrast to these findings reported in 1933 (4), Wooley and Armstrong state (1934) (5) that eleven of 113 sera (9.7 per cent) from individuals with no special contact with cases gave strong or moderate protection. This discrepancy will be discussed later.

Chronic Lethargic Encephalitis with Parkinson Sequelae.—Webster and Fite reported in 1933 (4) that sera from sixteen cases of lethargic encephalitis with Parkinson sequelae failed to protect mice against the virus. Similar results were reported by Levaditi, Schoen, and Levaditi (1934) (8) on sera from four cases. Wooley and Armstrong, however, state (1934) (5) that sera of four of twenty-nine (13.7 per cent) tested cases showed strong protection.

We have now studied a total of twenty-seven sera from typical cases. The results are negative. Nine cases gave a history of onset following an attack of influenza in 1918 to 1920. All have had Parkinson sequelae for 1 to 15 years.

Typical, Acute Encephalitis (Economo).—The negative effect of sera from the twenty-seven chronic cases of outspoken encephalitis of the Economo type raised the question of whether sera from the same disease in the more acute stages would show protective substances against the St. Louis virus. Realizing that a clinical diagnosis of this disease in the acute stages is often difficult, an effort was made to obtain sera from both typical and atypical cases.

Eight typical acute cases were tested and found to be negative. All gave histories of lethargy, ptosis or diplopia, tremors, and mononuclear pleocytosis of the spinal fluid. Two had beginning mask facies. Blood was drawn for testing 2, 2, 4, 6, 10, 12, 16, and 26 weeks after onset.

Atypical Primary Encephalitis.—Sixty-nine cases of atypical primary encephalitis were also tested with negative results. Symptoms and signs varied widely but were of such a nature in each case as to warrant a clinical impression of "encephalitis." Three cases occurred in 1932, twenty-eight in the autumn of 1933, thirty in 1934, and eight in 1935. Sera for testing were obtained 2 to 52 weeks after onset of illness. Twenty-four were from New York, two from New Jersey, six from Connecticut, two from Massachusetts, one from Pennsylvania, five from Maryland, four from Virginia, one each from Florida and Alabama, five from Illinois, four from Missouri, one from Indiana, eleven from Ohio, and two from California.

Japanese B Encephalitis.—We found (1934) (9) that sera from fifteen cases of Japanese B encephalitis did not protect against the St. Louis virus. These results were surprising in view of the reported similarity in epidemiological and clinical features of the Japanese and St. Louis diseases (10).

Sera were received from three persons supposed to have had the disease in August, 1924, aged 50, 51, and 60 years, and from nine persons convalescent from the August, 1933, epidemic, aged 17, 17, 20, 26, 33, 46, 53, 62, and 65 years. In these cases fever subsided 6 to 12 days after onset of symptoms. Sera were likewise received from three additional persons convalescent from the 1933 epidemic. Blood specimens were drawn from the 1924 cases about 10 years after onset and from the 1934 case about 4 months after onset. Specimens were tested after

about 6 weeks' aging *in vitro*. Each serum was tested twice with negative results. Kodama has recently confirmed these findings using Strain 3 virus and serum from convalescents in Japan (13). Further negative tests on sera from animals immunized with Takaki's B and A viruses (11), and futile attempts to establish the B virus from glycerinized material are reported below.

Postinfectious Encephalitis.—Sera from ten cases of postinfectious encephalitis were tested and found negative. Three cases were encephalitis following herpes zoster; two additional cases of herpes zoster without encephalitis were found negative. Four cases were post-measles encephalitis, two post-pertussis encephalitis, and one encephalitis complicating varicella.

Meningoencephalopathy, Indianapolis.—Sera from two convalescents from meningoencephalopathy at Indianapolis described by Kempf, Gilman, and Zerfas (12) did not protect against the virus (4).

Australian "X" Disease.—Serum from a case reported to have had "X" disease was found negative.

Poliomyelitis, Los Angeles, 1934.—Sera from eleven cases of poliomyelitis in Los Angeles, 1934, were obtained 4 weeks and 8 months after onset of symptoms. All were negative.

In summary, none of the 156 tested sera from persons believed to have had no exposure to St. Louis encephalitis has shown protective antibodies against the virus. Wooley and Armstrong's series of similar cases (*a*) lethargic encephalitis, (*b*) unclassified encephalitis, acute meningoencephalitis, epidemic meningoencephalopathy, poliomyelitis, traumatic encephalitis, postinfectious encephalitis, Jacksonian epilepsy, (*c*) other diseases not neural, and (*d*) normal controls with no special contact, gave positives at the rates of 13.7 per cent of 29, 11.4 per cent of 34, 13.1 per cent of 99, 9.7 per cent of 113, respectively, or 11 per cent of the total 275.

Results with Sera from Animals Immunized with Known Viruses

The protection test was used not only to determine the specificity of the reaction between serum and virus but to discover a serological relationship between the St. Louis and other known viruses. Sera from immunized animals known to protect against the homologous virus[2] were tested against the St. Louis virus. The results were nega-

[2] The herpes sera were supplied by Dr. Margaret Holden, and the Japanese encephalitis A and B sera by Professor I. Takaki. Dr. C. TenBroeck sent us the equine encephalomyelitis sera and carried out the protection tests with the encephalomyelitis virus. We are indebted to Dr. P. J. du Toit, Pretoria, South

tive, indicating no serological relation between this and the following viruses: herpes, Japanese B and A, poliomyelitis, equine encephalomyelitis, vesicular stomatitis, louping ill, blue tongue, and fox encephalitis.[2]

Herpes.—Sera from six rabbits immunized with the E. L. 1 Perdrau strain were tested with negative results.

Japanese Encephalitis A and B.—One specimen each of anti-A and anti-B goat serum was received for testing. The anti-A serum run on two occasions did not protect against the St. Louis virus. The anti-B serum contained preservative and gave some protection against St. Louis encephalitis, louping ill, and yellow fever viruses. When injected without virus into mice, it induced transient convulsions.

Besides sera, specimens of virus A and virus B were received. One lot of B virus in rabbit brain preserved in glycerin was received Feb. 8, 1934, and injected into six white-face mice and three 800 gm. rabbits intracerebrally, intracutaneously, and intracorneally. The animals remained well. Later, the material was injected into three 800 gm. rabbits intracorotidly, into three subdurally, into three intracerebrally, into two intratesticularly, and into two intravenously. All remained well. A second lot of B virus and a specimen of A virus were received May 11, 1934, and each injected subdurally, intracerebrally, intracarotidly, and intratesticularly into a total of twenty-five 800 gm. rabbits. No virus could be demonstrated.

Poliomyelitis.—Sera from two *Macacus rhesus* monkeys immunized by repeated injections of the M. V. virus did not protect against the St. Louis virus.

Equine Encephalomyelitis.—Sera from a rabbit immunized with the western strain and from a rabbit and horse immunized with the eastern strain were reported negative by Cox and Fite (14). Later tests have now been made with sera from a guinea pig immunized to the western strain and from a horse immunized to the eastern strain. These likewise showed no protective effect against the St. Louis virus. Here it should also be stated that sera from monkeys immunized with the St. Louis virus failed to protect guinea pigs against the two strains of encephalomyelitis virus (14).

Vesicular Stomatitis.—Serum from a rabbit immunized with the New Jersey strain and one from a rabbit immunized with the Indiana strain were reported negative by Cox and Fite (14). Serum from a monkey immunized with the St. Louis virus failed to protect guinea pigs against the vesicular stomatitis strains (14).

Louping Ill.—Serum from a horse convalescent from an experimental infection of louping ill showed no protective activity against the virus. In addition, sera

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Africa, for the louping ill and blue tongue sera, and to Dr. R. G. Green for the fox encephalitis sera.

from four persons showing specific protective substances against the louping ill virus (15) failed to protect against the St. Louis virus. Finally, anti-St. Louis monkey serum did not protect mice against the louping ill virus.

Blue Tongue.—Serum from sheep immunized with the routine passage virus said to be antigenically similar to all other known strains did not protect against the St. Louis virus.

Fox Encephalitis.—Sera from a normal fox and a fox immunized with fox encephalitis virus were negative.

Results with Sera from Persons with History of Exposure to the St. Louis Type of Encephalitis[3]

St. Louis Cases, 1933.—Sera from thirty-six cases on the encephalitis wards of the St. Louis City Isolation Hospital during the epidemic in August and September, 1933, were obtained for study. Eight cases diagnosed as not encephalitis of the prevailing type showed no protective properties in their sera. The remaining twenty-eight cases presented the clinical picture characteristic of the majority of cases of the epidemic (10); namely, high incidence among adults (17, 18, 22, 23, 28, 30, 31, 33, 36, 37, 38, 40, 49, 49, 50, 53, 55, 55, 60, 62, 64, 68, 75, and 75 years), systemic reactions including fever, headache, and vomiting, stiff neck and tongue tremors, and a lymphoid cell pleocytosis of spinal fluid. 82.5 per cent of the twenty-eight cases showed protective properties against the virus (Table IX). The negative cases were aged 23, 36, 40, 49, and 75 years. If the cases are grouped according to whether the first bleeding was made before or after the 14th day from onset, the seventeen sera drawn on the 14th day or later all protect, while of the eleven drawn less than 14 days from onset, only six, 54.5 per cent protect.

The negative effect of the "early" sera is not understood. The

[3] Sera from St. Louis cases of encephalitis, 1933, were obtained through the courtesy of Drs. R. S. Muckenfuss, J. Eschenbrenner, Jr., and S. Weisman. Dr. P. F. Stookey sent us sera from cases of encephalitis of the St. Louis and other types occurring in Kansas City, and brain tissue from fatal cases. Dr. H. D. McIntyre forwarded sera from sixteen cases of encephalitis in Cincinnati, 1933 and 1934. Sera from five cases of encephalitis in Paris, 1932, were sent by Dr. W. E. Conklin; later, sera from fifty cases of encephalitis of the St. Louis type in Paris, Danville, and Canton, Illinois, were sent by Dr. W. H. Tucker through the courtesy of Dr. F. J. Jirka. The Indiana State Board of Health kindly supplied sera from five cases of the disease.

view was taken in an earlier report that insufficient time had elapsed for the development of antibodies (4), but this is now untenable since second bleedings from these same cases 4 months after onset likewise gave negative results. Such explanations as inability of certain persons to elaborate demonstrable antibodies or incorrect diagnoses

TABLE IX

Sera Tested against Encephalitis Virus (St. Louis Type)

Diagnosis	No. tested	No. positive	Per cent positive
Healthy non-contacts	13	0	0
Chronic encephalitis Economo	27	0	0
Acute " "	8	0	0
Atypical, suspected encephalitis	69	0	0
Japanese B encephalitis	15	0	0
Postinfectious encephalitis	10	0	0
Meningoencephalopathy, Indianapolis	2	0	0
Australian "X" disease	1	0	0
Poliomyelitis, Los Angeles, 1934	11	0	0
Immunized animals	21	0	0
St. Louis not encephalitis, 1933	8	0	0
" " encephalitis, 1933	28	23	82 5
Kansas City encephalitis, 1933	4	3	75.0
Cincinnati " 1933	1	1	100.0
New York " 1933	2	2	100.0
Paris, Ill. " 1932	12	11	91.6
" " " 1934	9	6	66.6
Danville, Ill. " 1934	21	14	66.6
Canton, Ill. " 1934	6	3	50.0
Indiana " 1934	5	5	100.0
Cincinnati " 1934	4	2	50.0
California " 1934	3	1	33.3

appear most probable, even though the negative reactors were concentrated in the less than 14 day group.

These results are not in complete agreement with those of Muckenfuss (6). Fifteen of the thirty-four sera were tested by both workers with agreement in the case of eleven and in the case of four, positive results by Muckenfuss and negative by ourselves, a disagreement of 26.6 per cent. We tested two of the disputed sera three times on two specimens; the other two, once only.

Kansas City Cases, 1933.—Encephalitis, so conspicuous in St. Louis during August and September, 1933, was present at the same time in Kansas City. Here, however, fewer cases were recorded and the clinical pictures were more varied. From one of two tested fatal cases, a virus was recovered similar to that obtained from fatal cases in St. Louis (2). At the same time sera from four outspoken cases were tested against the St. Louis virus and three found positive (4).

Ohio Case, 1933.—A white male, aged 41, contracted encephalitis August 30, in Cincinnati, of a type similar to the St. Louis disease. His serum, collected and tested 1 year later, October, 1934, neutralized the virus.

New York Cases, 1933.—Recognition of cases of the St. Louis type of encephalitis naturally became more difficult in places remote from the epidemic. Tests were made, therefore, on sera from various forms of atypical encephalitis, sixteen in all, from September to the end of December, 1933. Of these, only two were considered clinically as possible cases of the St. Louis disease.

No. 47. M. S., a white male, aged 27, was admitted to St. Luke's Hospital, Dr. Frissell's service, on Sept. 9, 1933, with a 3 day history of nausea, vomiting, fever, malaise, generalized throbbing headache, and no history of having been to St. Louis or having come in contact with anyone from St. Louis. On admission his temperature was 101°F. There was a slight leucocytosis, 10,900, 80 per cent polymorphonuclears. The Wassermann reaction was negative. The other findings were chiefly neurological and consisted of absent abdominal reflexes, hyperactive tendon reflexes, medium coarse nystagmus, soreness but no stiffness of neck a suggestive Kernig, and weakness of flexion of right arm. Spinal fluid, pressure 240; cells 144 (lymphoid cells 96 per cent), globulin +, protein 82, sugar 58. Within a week's time all symptoms and signs had returned to normal, save for hyperactivity of tendon reflexes and persistence of nystagmus. The cell count of the spinal fluid had dropped to 50 in 3 weeks, and to 15 in 6 weeks.

Dr. Frissell called this case to our attention as possible St. Louis encephalitis and tests on sera drawn 2 and 6 months after onset, ten in all, were strongly positive (4), (Table X).

No. 63. B., a white female, aged 39. After 3 weeks' vacation in Kentucky, returned to New York with severe headache. She was seen by Dr. H. T. Chickering on Sept. 12, 1933. Her temperature was 103°F. She complained of severe headache, different from any other, accompanied by chills. This condition cleared up rapidly and she was discharged as well on Sept. 25.

Dr. Chickering mentioned this case as possible mild encephalitis of the St. Louis type. Two tests on serum drawn 11 weeks after onset were positive (4), (Table X).

Paris, Illinois, Cases, 1932.—In searching for a possible relationship between the 1933 St. Louis and previous outbreaks of encephalitis, mention has been made of protection tests run on sera from cases of Economo encephalitis with onset following influenza in 1918, and meningoencephalopathy in Indianapolis. The results were negative. The case of the outbreak of encephalitis in Paris, Illinois, in 1932, however, was different. Encephalitis appeared in Paris, Illinois, a

TABLE X

Positive Protection of Sera from New York, 1933, Cases against the St. Louis Virus

Serum No.	Test No	Date of test	Virus-serum dilution				Diagnosis
			10^{-2}	10^{-4}	10^{-6}	10^{-8}	
		1933					
47	8	Nov. 2	6, 7, S, S	6, S, S, S	S, S, S, S	S, S, S, S	Acute encephalitis (St. Louis?)
46	8	" 2	4, 4, 4, 6	5, 5, 5, 5	5, 5, 6, 6	6, 6, 7, 9	Chronic encephalitis (Parkinson)
47	12	" 16	5, 6, 7, 7	7, 7, 9, 10	S, S, S, S	S, S, S, S	Acute encephalitis (St. Louis?)
H			4, 5, 5, 6	5, 5, 6, 6	5, 6, 6, 6	5, 5, 7, 9	Normal non-contact
63	13	Nov. 23	5, 6, 6, 7	6, 7, 7, 7	S, S, S, S	S, S, S, S	Acute encephalitis (St. Louis?)
58			5, 5, 5, 7	5, 5, 6, 8	5, 5, 6, 6	6, 7, 9, 10	Chronic encephalitis (Parkinson)

S = mice remained well 21 days.

community of about 9000 persons, in July and August, 1932 (16, 10). Twenty-seven persons were affected, aged 33 to 80, with only three under 50. The mortality was close to 50 per cent, but convalescents were relatively free of sequelae. The outstanding symptoms recorded were headache, fever, nausea and vomiting, diplopia, and delirium or stupor. Facies were set, neck rigid, and tongue tremorous. Sera from five convalescents drawn 15 months after onset were tested against the St. Louis virus. Four of the five gave definite protection (4), (Table XI). Later, February, 1935, 2½ years after the outbreak,

TABLE XI

Protection Tests on Sera from Cases in Paris, Illinois, 1932 and 1934

Serum	Test	Virus-serum dilution				Result
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	
C-non-contact	47	5, 5, 6, 6	6, 6, 6, 6	5, 7, 7, 7	8, 8, S, S	0
Monkey immune		6, 6, 8, 8	6, 7, 8, S	S, S, S, S	S, S, S, S	+
246 Paris '31		4, 5, 5, 5	6, 6, 7, 9	6, 7, 7, 9		0
242 " '32		9, 10, S, S	S, S, S, S	S, S, S, S		++
248 " '32		9, 9, 9, S	S, S, S, S	S, S, S, S		++
247 " '32		7, 9, 9, 9	S, S, S, S	S, S, S, S		++
241 " '32		5, 5, 8, S	6, 7, 9, 9	S, S, S, S		+
239 " '34		6, 6, 6, 7	6, 8, 9, S	S, S, S, S		+
244 " '34		6, 7, 7, 8	6, 6, 7, 7	S, S, S, S		+
240 " '34		6, 7, 8, 8	5, 7, 9, 9	10, S, S, S		+
243 " '34		6, 6, 6, 7	6, 6, 9, 9	6, 7, 7, 8		0
245 " '34		4, 5, 5, 6	6, 6, 6, 6	6, 6, 6, 6		0
C-non-contact	48	5, 5, 5, 5	5, 5, 5, 6, 6, 7	6, 6, 7, 7, 7, 9	7, 8, 8, 9	0
251 Paris '32		6, 8, 8, 8	S, S, S, S, S, S	S, S, S, S, S, S		++
250 " '32		5, 7, 8, 8	6, 9, S, S, S, S	S, S, S, S, S, S		++
257 " '32		7, 8, 8, 9	9, 9, 9, S, S, S	S, S, S, S, S, S		+
252 " '32		5, 5, 8, 8	5, 6, 7, 7, 8, 8	6, S, S, S, S, S		+
253 " '32		5, 5, 6, 7	6, 6, 6, 7, 8, 8	9, S, S, S, S, S		+
256 " '32		5, 5, 5, 5	5, 6, 6, 7, 8, 8	8, 8, 8, 8, 8, S		0
258 " '34		7, 8, 8, 8	9, S, S, S, S, S	S, S, S, S, S, S		++
249 " '34		5, 7, 8, 9	9, S, S, S, S, S	8, S, S, S, S, S		++
255 " '34		6, 7, 7, 8	7, 8, 8, 8, 8, 8	7, S, S, S, S, S		+
254 " '34		6, 6, 8, 8	6, 7, 7, 9, 9, 9	7, 8, 8, S, S, S		+

S = mice remained well 21 days.
Blank spaces indicate dilution not tested.

additional sera were obtained from ten of the cases, including three of the five previously tested. Nine of the ten or eleven of the total twelve (91.6 per cent) were positive (Table XI). Wooley and Armstrong found ten of eleven (90.9 per cent) positive (5).

Illinois, Ohio, and Indiana Cases, 1934.—The reappearance of encephalitis in the north central states during the summer of 1934 afforded another opportunity to study the specificity of the serum reaction. Cases clinically resembling the St. Louis type were occurring in localized outbreaks in Illinois and sporadically in Ohio and Indiana.

Paris, Illinois.—Nine convalescents, aged 10, 15, 33, 35, 38, 44, 68, 70, and 76 years, were bled 5 to 6 months after onset of symptoms and their sera tested against the St. Louis virus. Six of the nine specimens, 66.6 per cent, gave definite protection. The negative cases were aged 10, 38, and 70 years. If the 10 year old case is omitted, the percentage of positives becomes 75.

Danville, Illinois.—Twenty-one convalescents, aged 9, 10, 15, 16, 19, 20, 22, 26, 26, 27, 30, 30, 31, 31, 32, 33, 36, 38, 40, 59, and 60 years respectively, were tested 2 months after onset of symptoms. Fourteen of the twenty-one, 66.6 per cent, showed protective properties in their sera. The negative cases were aged 9, 10, 15, 16, 22, 38, and 40 years respectively. If the four cases aged 16 years or less are omitted in the count, the ratio of positive reactors is increased to fourteen of seventeen, or 82.5 per cent.

Canton, Illinois.—Sera were obtained from six convalescents aged 7, 12, 51, 58, 75, and 80 years. Those aged 58, 75, and 80 years protected (50 per cent). If the 7 and 12 year cases are omitted from the count, the ratio of positives becomes three of four (75 per cent).

In summary, of thirty-six Illinois 1934 cases tested, seven were aged 16 years or less and did not protect; of the twenty-nine remaining however, twenty-three (79.3 per cent) were positive.

Indiana Cases.—Sera from five cases drawn at least 4 weeks after onset were tested and found to protect against the virus.

Ohio Cases.—Sera from two of four typical cases drawn 1 to 3 months after onset neutralized the virus.

California Cases, 1934.—Sera from one of three cases protected against the St. Louis virus.

DISCUSSION

The specificity of the encephalitis protection test is indicated by the present work and by reports of Wooley and Armstrong and Muck-

enfuss. The latter workers, however, record a 10 to 30 per cent incidence respectively of positive reactors among groups of persons with no special exposure to the St. Louis disease. This discrepancy is due either to differences in batches of sera tested, or more probably to differences in technique and criteria for testing. Our procedure renders it unlikely that a negative serum would be called positive but admits the possibility of a few weakly positive sera being called negative. Be that as it may, the specificity of the serum-virus reaction is further evidence that this virus is the specific agent responsible for the human disease, and finally, that the virus is different and the encephalitis in human beings is serologically distinct from others previously described.

Knowledge that antibodies persist for 2½ years in the blood of convalescents is an aid in searching for an endemic focus and in mapping out the time and space spread of the virus. Thus far, we know that the disease appeared in Paris, Illinois, in 1932, and was present in 1933 and 1934 in the north central states and New York.

CONCLUSIONS

1. A protection test for measuring serological protective properties against the encephalitis (St. Louis type) virus is described.

2. Normal non-contact sera and sera from persons supposed to have had no exposure to the disease do not protect against the virus. 82.5 per cent of sera from tested St. Louis encephalitis convalescents and at least 66 per cent of sera from tested persons thought to have had the disease do show protective properties.

3. The protective activity of sera is maintained for at least 2½ years after onset of the disease. *In vitro* aging of serum decreases its activity.

4. Protection tests indicate that the virus was present as early as 1932 in Paris, Illinois, spread through the north central states and reached New York in 1933, and was again active in the north central states in 1934.

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SEROLOGICAL TESTS WITH AMINO ACIDS

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In the course of studies on the immunological specificity of peptides (1, 2) it was found necessary to establish to what extent amino acids which are contained in proteins can be differentiated by immune sera.[1]

PREPARATION OF PARA-NITRO- AND PARA-AMINOBENZOYL DERIVATIVES OF AMINO ACIDS

Nitrobenzoylation of the amino acids was mostly carried out by the method used previously for the preparation of p-nitrobenzoyl peptides (2). In the case of histidine a dilute solution of sodium hydroxide was used instead of sodium bicarbonate (3). Phenylalanine and tryptophane were dissolved in a small amount of normal sodium hydroxide before adding sodium bicarbonate. For the nitrobenzoylation of glutamic and aspartic acid the directions given by E. Fischer for the preparation of the benzoyl derivatives were followed (4). The products were freed from nitrobenzoic acid by extraction with ether, and if the nitrobenzoyl amino acid was appreciably soluble in ether, the ether solution was concentrated to a small volume and the substance precipitated by addition of petroleum ether (boiling point 90 to 100°).

p-Nitrobenzoyl-l-tyrosine. Of *l*-tyrosine ethylester hydrochloride, 2.45 grams were dissolved in 5 cc. of water and 20 cc. of chloroform added. After cooling to −10°C. and addition of 2 cc. 5 N sodium hydroxide one-half of a solution of 3.7 gram of *p*-nitrobenzoyl chloride in 20 cc. chloroform was added in 5 portions with vigorous shaking at low temperature. After addition of 10 cc. N sodium carbonate and the remainder of *p*-nitrobenzoyl chloride the mixture was shaken until no more carbon dioxide was given off. The chloroform layer was sepa-

[1] See Landsteiner, K., Die Spezifizität der serologischen Reaktionen, Springer, Berlin, 1933; Immunchemische Spezifizität, Reale Accademia d'Italia 1934–VII.

rated, dried over anhydrous sodium sulfate and concentrated to a small volume. The *p*-nitrobenzoyl tyrosine ester was precipitated by addition of petroleum ether, yield 2.9 gram. It was recrystallized by dissolving in 3 parts of absolute alcohol and diluting the solution with an equal volume of water. Clusters of long needles, melting point 140 to 141°. Analysis: Calculated for $C_{18}H_{18}O_6N_2$:N 7.82, found 7.75. A solution of 1.79 gram of *p*-nitrobenzoyl-*l*-tyrosine ester in 10 cc. of N sodium hydroxide was kept at 15°C. for fifteen minutes and was then filtered and made acid to Congo red by addition of concentrated hydrochloric acid. The oil which separated crystallized upon rubbing, yield 1.55 gram. It was recrystallized from 40 parts of water. Needles, melting point 163 to 164°. Analysis: Calculated for $C_{16}H_{14}O_6N_2$:N 8.48, found 8.43.

p-Nitrobenzoyl-glycine and p-nitrobenzoyl-d,l-leucine have been described in a former publication (1); the other *p*-nitrobenzoyl amino acids prepared were:

p-Nitrobenzoyl-d,l-alanine. Recrystallized from dilute alcohol. Platelets, melting point 193 to 194°. Analysis: Calculated for $C_{10}H_{10}O_5N_2$:N 11.76, found 11.53.

p-Nitrobenzoyl-d,l-valine. Recrystallized from 50 per cent alcohol. Platelets, melting point 168 to 170°. Analysis: Calculated for $C_{12}H_{14}O_5N_2$:N 10.53, found 10.51.

p-Nitrobenzoyl-d,l-phenylalanine. Recrystallized from absolute alcohol. Platelets, melting point 166 to 168°. Analysis: Calculated for $C_{16}H_{14}O_5N_2$:N 8.92, found 8.86.

p-Nitrobenzoyl-l-tryptophane. Recrystallized from 50 per cent alcohol. Platelets, melting point 116 to 117°. Analysis: After drying at 78°C. in vacuo over H_2SO_4; calculated for $C_{18}H_{15}O_5N_3$:N 11.90, found 11.72.

p-Nitrobenzoyl-l-histidine (3). Melting point 251 to 252°. Analysis: Calculated for $C_{13}H_{12}O_5N_4$:N 18.42, found 18.18.

p-Nitrobenzoyl-l-aspartic acid. Recrystallized from water. Needles, melting point 150 to 151°. Analysis: After drying at 78°C. in vacuo over H_2SO_4; calculated for $C_{11}H_{10}O_7N_2$:N 9.93, found 9.68.

p-Nitrobenzoyl-d,l-aspartic acid. Recrystallized from water. Platelets, melting point 159 to 161°. Analysis: After drying at 78°C. in vacuo over H_2SO_4; calculated for $C_{11}H_{10}O_7N_2$:N 9.93, found 9.85.

p-Nitrobenzoyl-l-glutamic acid. Recrystallized from water. Platelets, melting point 112 to 113°. Analysis: After drying at 78°C. in vacuo over H_2SO_4; calculated for $C_{12}H_{12}O_7N_2$:N 9.46, found 9.14.

p-Nitrobenzoyl-d,l-glutamic acid. Recrystallized from water. Plate-

lets, melting point 95 to 96°. Analysis: After drying at 78°C. in vacuo over H_2SO_4; calculated for $C_{12}H_{12}O_7N_2$:N 9.46, found 9.35.

The aminobenzoyl amino acids were obtained by reduction of the nitrobenzoyl compounds by means of ferrous sulfate (see (2)).

p-Aminobenzoyl-d,l-alanine. After reduction of the nitrobenzoyl compound the filtered solution was evaporated to dryness in vacuo, and the residue was dissolved in 10 parts of water. The solution was made weakly acid to Congo red by addition of hydrochloric acid and kept in the icebox overnight. The precipitate was recrystallized from 10 parts of water. Yield 2.4 gram from 3.6 grams of nitrobenzoyl compound. Clusters of needles, melting point 194 to 195°. Analysis: Calculated for $C_{10}H_{12}O_3N_2$:N 13.46, found 13.55.

p-Aminobenzoyl-d,l-valine. The solution resulting from reduction of the nitro compound was evaporated in vacuo to a small volume and made weakly acid to Congo red. The precipitate was filtered off after cooling. The aminobenzoyl compound was dissolved in normal hydrochloric acid and after removal of a small amount of insoluble material was reprecipitated by addition of normal sodium hydroxide. It was recrystallized from 100 parts of water. Yield 4.4 grams from 6.4 grams of nitro compound. Long needles, melting point 196 to 197°. Analysis: Calculated for $C_{12}H_{16}O_3N_2$:N 11.86, found 12.02.

p-Aminobenzoyl-l-tryptophane. To the concentrated solution 10 volumes of alcohol were added, ammonium sulfate was filtered off, the filtrate evaporated to dryness in vacuo, the residue was dissolved in a small volume of water and the substance precipitated by addition of hydrochloric acid to weak acidity to Congo red. It was purified by dissolving in normal hydrochloric acid and reprecipitation from the filtered solution by addition of alkali, and was recrystallized from 200 parts of water. Yield 1.6 grams from 3.3 grams of nitro compound. Clusters of needles, melting point 210 to 211°. Analysis: Calculated for $C_{18}H_{17}O_3N_3$:N 13.00, found 12.70.

p-Aminobenzoyl-d,l-aspartic acid. The procedure described for *p*-aminobenzoyl-*l*-tryptophane was followed and the substance was recrystallized from 10 parts of water. Yield 2 grams from 3.4 grams of nitro compound. Irregular platelets, melting point 193 to 194°. Analysis: Calculated for $C_{11}H_{12}O_5N_2$:N 11.11, found 10.95.

p-Aminobenzoyl-l-glutamic acid. After removal of ammonium sulfate and concentration in vacuo the aminobenzoyl compound was precipitated as a copper salt from a neutral aqueous solution by the addition of copper sulfate. The copper salt was washed several times

with water, suspended in a small amount of water and decomposed with hydrogen sulfide. The aminobenzoyl-glutamic acid crystallized from the filtered solution upon cooling. It was recrystallized from water. Yield 2.7 grams from 4.4 grams of nitrocompound. Platelets, melting

TABLE 1

To 0.2 cc. of the 1:500 diluted antigens in terms of a 5 per cent stock solution (prepared with chicken serum) were added 2 capillary drops of immune serum

Readings were taken after one hour at room temperature—first line—and after standing over night in the icebox—second line.

IMMUNE SERA FOR	TEST ANTIGENS PREPARED FROM CHICKEN SERUM AND THE AMINOBENZOYL DERIVATIVES OF							
	Glycine	*d,l*-Alanine	*d,l*-Valine	*d,l*-Leucine	*d,l*-Aspartic acid	*d*-Glutamic acid	*d,l*-Glutamic acid	*l*-Tryptophane
Glycine no. 1	++±	+±	0	0	0	0	0	0
	+++	+±	0	0	0	0	0	0
Glycine no. 2	+±	±	0	0	0	0	0	0
	++±	+	0	0	0	0	0	0
d,l-Leucine no. 1	0	0	+	++	0	0	0	0
	0	tr.	+±	++±	0	0	0	0
d,l-Leucine no. 2	0	±	+±	++	0	0	0	0
	0	±	+±	++±	0	0	0	0
d-Glutamic acid no. 1	0	0	0	0	±	++	+±	0
	0	0	0	0	±	++±	++	0
d-Glutamic acid no. 2	0	0	0	0	tr.	++	+±	0
	0	0	0	0	±	+++	++±	0
l-Tryptophane	0	0	0	0	0	0	0	+±
	0	0	f.tr.	f.tr.	0	0	0	++

point 172° to 173°. Analysis: Calculated for $C_{12}H_{14}O_5N_2$:N 10.53, found 10.46.

p-Aminobenzoyl-d,l-glutam c acid. It was isolated from the solution by the procedure described for *p*-aminobenzoyl-*l*-tryptophane and was recrystallized from 20 parts of water. Yield 1.8 grams from 2.6 grams of nitrocompound. Long narrow platelets, melting point 198 to 199°. Analysis: Calculated for $C_{12}H_{14}O_5N_2$:N 10.53, found 10.18.

SEROLOGICAL TESTS

Preparation of antigens. The antigens were prepared from the amino-benzoyl amino acids as described previously (1). The azoproteins used for immunization were purified by redissolving in water with the aid of dilute alkali, reprecipitation with acid and repeated washings with saline. The antigens used for the tests were made from chicken serum (1).

Immunization and tests. Rabbits were immunized by intravenous injections of 2 cc. of a solution containing 10 mgm. of antigen in 1 cc. as described previously (5). With the inhibition tests it is necessary to

TABLE 2

For the inhibition tests 0.2 cc. of chicken serum antigens (diluted 1:500) was mixed with 0.05 cc. of a neutral solution of the nitrobenzoyl derivatives of the amino acids (concentration 0.25 millimol in 10 cc.) or, in the control tube, 0.05 cc. saline, and 2 capillary drops of homologous immune serum were added

Readings were taken after fifteen minutes—first line—and after 1 hour at room temperature—second line.

IMMUNE SERA FOR	GLYCINE	d,l-ALANINE	d,l-VALINE	d,l-LEUCINE	d,l-PHENYL ALANINE	l-TYROSINE	l-TRYPTO-PHANE	l-HISTIDINE	l-ASPARTIC ACID	d,l-ASPARTIC ACID	d-GLUTAMIC ACID	d,l-GLUTAMIC ACID	CONTROL
Glycine	0	+	+±	+±	+±	+±	++	+±	+±	+±	+±	+±	++
	f.tr.	+±	++	++	++	+±	++	++	++	++	++	++	++
d,l-Leucine	+±	+	tr.	0	tr.	±	±	+±	+±	+±	+±	+±	++
	++	++	+	0	+	++	+±	++	++	++	++	++	++±
d-Glutamic acid	+±	+±	+	+	+	+	+	+	+	+	0	0	+±
	+±	++	++	+±	+±	+±	++	+±	+±	+±	0	0	++

determine a suitable concentration of the solutions; in the experiment given below 0.25 millimol in 10 cc. were used. For the recording of the results see (1).

The results of the precipitin tests are presented in table 1. The tests were performed with antigen concentrations from 1:100 to 1:12500, but only those made with the concentration 1:500 are included in the table, since these reactions were the strongest and the other tests showed the same degree of specificity.

Inhibition tests made with the homologous and other amino acids are summarized in table 2.

From the tables it is seen that in general various amino acids are readily distinguishable by serological tests and that distinct cross-reactions occur only between related amino acids, namely—in our experiments—glycine and alanine; leucine, valine and alanine; glutamic acid and aspartic acid. In the inhibition tests some effect on the reaction of leucine immune serum was shown by the nitrobenzoyl derivatives of tryptophane, tyrosine and phenylalanine.

Putting together the experiments reported previously on peptides containing glycine and leucine only (1, 2), and the present results it follows that a large number of serologically different peptides can be built up if one uses all the amino acids that occur in proteins.

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PATHOLOGY OF PNEUMOCOCCUS INFECTION IN MICE FOLLOWING INTRANASAL INSTILLATION

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PLATES 3 TO 5

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Webster and Clow (1) have shown that mice raised under controlled conditions present marked individual responses to pneumococcus infection; for example, a complete refractory state, a prolonged carrier condition, cervical adenitis with or without fatal termination, fatal pneumonia, or death from septicemia without any localization. Moreover, Webster had pointed out in an earlier paper (2) that individual breeds of mice behave in a predictable way and differently towards intranasal infection.

A study of the pathological changes in these mice has seemed warranted in order to determine not only the individual differences in mice belonging to one breed, but also the differences in the picture produced in the different breeds by the same strain of pneumococci and the reaction of identical groups of mice all of one breed to infection with pneumococci belonging to different types. The work to be presented shows that, contrary to previous reports, provided strains of pneumococci be selected on the basis of their intranasal as distinct from their intraperitoneal virulence, and the balance of virulence of organisms on the one hand and resistance of the mice on the other be so chosen that the mice succumb in not less than 4 days, a pneumonia can be evoked in mice which have received no preparatory treatment.

In 1924 Stillman and Branch reported the production of pneumococcus pneumonia in alcoholized mice (3) and gave a description of the lesions produced (4). The percentage of mice showing evidences of localization in the lung in the groups previously immunized with homologous or even heterologous pneumococci was far greater than in the non-immunized groups. Congestion of the interalveolar capillaries was the first lesion they noted, and this was often associated with a serofibrinous pleurisy without any other lesion in the lung. The congestion was

followed by interstitial inflammation of the alveolar walls and dilatation of the perivascular lymphatics which contained cell debris; later, the alveoli contained red cells and polymorphonuclears with a little fibrin. The most advanced stage showed a leucocytic exudate and extreme anemia of the alveolar walls. The initial lesion was in the alveoli and spread occurred both centripetally and centrifugally.

Stillman and Branch studied the inception of the lesion (5) and noted, 6 hours after spraying the animal with pneumococci, small areas of interstitial inflammation of the alveolar wall with a very slight amount of exudate.

Griffith (6) twice noted consolidation of the lungs and bilateral pleural effusion in mice dying with Type II pneumococcus infection after having been immunized beforehand with Type I serum, and also a grey consolidation of one lobe in a mouse succumbing to a Type I pneumococcus of low invasiveness. He gives no account of a histological study.

More recently, Neufeld and Kuhn (7) have described pneumonia in etherized mice infected intranasally with Type I and Type XIX strains of pneumococcus. Unless the mice were anesthetized when the inoculation was made, pneumonia did not develop, save in two cases in which the mice had been infected intranasally daily for 2 weeks with a Type XIX strain. The histological picture of the Type XIX pneumonia, briefly described, is said to be similar to the picture in experimental pneumonia in the monkey (8) and in human lobar pneumonia. Hoyle (9) also reports the production of all stages from mild congestion to severe pneumonia in etherized mice infected intranasally with pneumococci.

Little has been published on pathological changes in the other organs of mice following pneumococcus infections. As stated above, Webster and Clow (1) noted cervical adenitis, and Neufeld and Etinger-Tulczynska (10) also noted swelling of the whole face in mice infected with Type I organisms, a condition which, as will be pointed out below, is probably secondary to a cervical adenitis.

Material and Technique

For our experiments several inbred breeds of mice and many different strains and types of pneumococci were used. All strains of pneumococci used were freshly isolated from human cases of pneumonia. It is important to note at this point that, as Griffith (6) and Webster and Clow (1) have shown, not all strains are suitable for certain phases of the work, especially for those having to do with intranasal virulence and the production of pneumonia. A strain may be of maximum intraperitoneal virulence and yet fail to produce death or disease even when introduced into the noses of highly susceptible mice. The converse, high intranasal and low intraperitoneal virulence, is also found though the intraperitoneal virulence can never be very low. Thus, strains of moderate intraperitoneal virulence may have a high intranasal virulence, whereas strains of very low intraperitoneal virulence do not kill when introduced into the nose. The use of inbred strains of mice which will react in a predictable manner and of

carefully selected strains of pneumococci has proved of the utmost importance. Neglect of these two essentials will explain many of the inconsistencies and failures of the past. Observance of them provides the delicate balance between organism and host, which Wadsworth (11) deems essential to the consistent production of pneumonia. It is often necessary to test as many as 50 or 60 strains of pneumococci to obtain one or two with an appreciable intranasal virulence even for highly susceptible mice, and this is especially true when Type III strains, which are most frequently intranasally virulent, are excluded.

In the case of each breed it was possible, after a few preliminary experiments, to predict within certain limits the mortality rate and the incubation time following the intranasal inoculation of a given strain of pneumococci. The technique of inoculation has already been described (1). The inoculated mice were placed in separate cages and kept under observation for the next 2 or 3 weeks. Whenever possible, a postmortem examination was performed immediately on death and animals in a moribund condition were sacrificed for this purpose, though no mice were killed until there was a certainty that they would die shortly. In some cases, death occurred during the night and postmortem changes had set in when the autopsy was performed. A note was always made to this effect. Cultures of the heart's blood were taken at autopsy. All gross lesions were noted, and besides the lungs, the kidneys, liver, spleen, and cervical lymph nodes were taken for section and fixed in Zenker's fluid. A tube was passed down the trachea, the lungs filled with fixative under gentle pressure, the tube withdrawn, the trachea tied off, and the lungs fixed *in toto* before removal. Blocks of both were so prepared as to yield sections containing a portion of each lobe. In this way, a representative picture was obtained of both lungs. In a few cases serial sections were made. Sections were stained in eosin-methylene blue or hematoxylin and eosin.

While chief attention has been paid to the pneumonia and its development, the lesions in the other organs have been studied as well. The general pathological picture will be described first, both in relation to the different breeds of mice and to the different types of pneumococci.

Lesions in Different Breeds of Mice

Numerous scattered observations in this laboratory had shown that the lesions produced in different breeds of mice following the intranasal inoculation of one and the same strain of pneumococci differed from breed to breed. Experiments were undertaken to bring out this point.

Twenty albino resistant mice and twenty albino susceptible mice, both of Institute stocks, were each given an intranasal inoculation of 0.02 cc. of a 1/100

dilution of an 18 hour culture of Type III pneumococcus obtained from the heart's blood of a mouse dying of an intranasal inoculation with this organism.

The mortality was high in the susceptible group—65 per cent—, and low in the resistant group,—25 per cent. The susceptibles, moreover, were the first to succumb, and for those that died in this group the average survival time was only 72.5 hours compared with 120.8 hours in the resistants.

An examination of the sections prepared from the different organs revealed the fact that there was a difference in the manner in which the two breeds of mice reacted to the intranasal inoculation of the same strain of microorganism. The circumstances under which the test was made give assurance that the only variable factor lay in the breed of mice inoculated.

Lungs.—The lungs from the two groups showed quantitative differences. Macroscopically, the resistant mice showed no changes while three of the susceptible group showed consolidation and one showed serofibrinous pleurisy. In the resistant group, despite the fact that survival time was longer, all five mice showed very mild lesions microscopically. All showed congestion and early dilatation of the perivascular lymphatics, in four cases the channels containing only fluid and a few organisms. In the fifth, there were also polymorphonuclears and debris. One case showed very early infiltration of the alveolar walls in a small area and another a little fluid exudate into the alveoli.

In the susceptible group the changes were definitely more marked, but in eleven out of the thirteen cases could still be described as mild. There were congestion and perivascular lymphatic involvement, with mononuclear cells in the dilated lymph vessels. Three cases showed moderately large areas of exudate both interstitially in the alveolar walls where it consisted of leucocytes, and in the alveoli, consisting chiefly of fluid and red cells with a few polymorphonuclears. In one mouse which died in 75 hours the changes were similar in nature, but considerably more advanced.

Cervical Nodes.—The changes in the lymphatic system were next in importance after those in the lung. The lymph nodes from parts of the body other than the cervical region, except those at the hila of the lungs and occasionally those of the pre-aortic group, showed no changes. A pelvic node was frequently included in the section of the kidney, and was invariably normal. In the resistant group the changes in the cervical nodes were always conspicuous. The nodes,—which in the gross were large and opaque,—proved microscopically to be the site of large abscesses which completely or almost completely replaced the normal structure. This lesion was present in every mouse. In the susceptible group nine of the thirteen showed changes. Macroscopically, the nodes appeared

enlarged, but in only one case were they opaque. In four, the microscopic lesions were mild and consisted only of a dilatation of the peripheral sinuses which contained numbers of mononuclear cells and a few organisms. In five cases there were abscesses similar to those found in the resistant group.

Spleen.—The striking picture in the spleens of the resistant group is the necrosis or abscess formation in the follicles. In four of the five mice there was abscess formation in most of the follicles. In the susceptible group the lesion was less advanced than in the resistant group. In ten of the thirteen there were changes in the follicles, and in seven of these the lesion amounted to abscess formation. One spleen was normal and the other two showed an increased number of mononuclear cells in the pulp.

Kidneys.—Normal in both groups.

Liver.—Few lesions in any of the mice. However, two resistant mice showed thromboses of the branches of the portal vein with infarcts of the liver.

An attempt was made to repeat this study of the lesions in different breeds of mice with other than Type III pneumococci. The difficulty encountered was to find a strain which would kill resistant albino mice when inoculated intranasally. One intranasally virulent strain was obtained, however.

An 18 hour culture of a Type XIX strain, which had proved highly virulent for Swiss mice when inoculated intranasally, was given intranasally to 20 resistant albinos and 20 susceptible albinos. The technique followed was that already given. The mortality rate and average survival time of the susceptible group was 55 per cent and 77.7 hours. In the resistant group only three died (mortality 15 per cent), and all of these died early (survival time 52.3 hours).

In the resistant group there was no evidence of localization in the lungs. One mouse showed a bilateral serofibrinous pleurisy, but there were no demonstrable lesions in the underlying lung. The other two showed only capillary congestion. In two of the three there were slight changes in the kidneys—granular debris in Bowman's capsule and convoluted tubules and some dilatation of the convoluted tubules. The other organs were normal.

In the susceptible group eight of the eleven mice showed some evidence of localization in the lungs. Of these, four had shown consolidation recognizable macroscopically. In three there was only capillary congestion. Four of the other eight showed only slight interstitial accumulation of polymorphonuclears and monocytes with dilatation of the perivascular lymphatics which contained fluid and a few cells. In the other four mice the lesions were advanced. They consisted of interstitial accumulation of cells and fluid in the alveolar septae, exudate of fluid with some red cells and polymorphonuclears into the alveoli, which was in some instances lobar in extent, some dilatation of the perivascular lymphatics which contained fluid and some cells, and in two cases a bilateral serofibrinous pleural exudate. In the other organs the changes were slight, save in

the kidney. Two of the eleven mice showed a frank diffuse nephritis with fibrin thrombi in the glomerular loops, changes in the cells of the convoluted tubules which contained colloid droplets or were frankly necrotic, dilatation of these tubules, and granular casts. In four others there were moderate tubular changes. In one case, the liver showed many areas of necrosis, and in two there were very small areas of necrosis in the splenic pulp.

In summary, of the lesions in the two breeds of mice, it can be said that there were definite quantitative differences in the two breeds (Table I). Thus, in the experiment with Type III organisms, the resistant mice showed very slight changes in the lungs, but marked lesions in the cervical nodes and follicles of the spleen. The susceptible mice showed more advanced lesions in the lungs, but less marked

TABLE I

Breed of mice	Type of pneumococcus	Lesion in organs		
		Lungs	Lymphatic system	Kidneys
Resistant	III	± (interstitial)	++	0
Susceptible	III	++ (interstitial)	+	0
Resistant	XIX	0	0	±
Susceptible	XIX	++ (interstitial)	0	+ (glomerular)
Unselected	I	++ (diffuse)	±	++ (glomerular)
Unselected	II	++ (confluent)	±	+ (tubular)
Unselected	III	++ (interstitial)	++	0

changes in the lymphoid tissue of the cervical nodes and spleen. In the experiments with Type XIX organisms the differences in the lungs were even more striking, although the small number of resistant mice which succumbed might raise the question as to the significance of the differences in this one particular case. Thus, none of the three resistant mice showed any local reaction in the lung, though one showed serofibrinous pleurisy. On the other hand, eight of the eleven susceptible mice showed local reaction in the lung, and in four of these the changes were advanced. It was impossible to be certain of differences in other organs, but it was possible that the susceptible group showed a higher incidence of nephritis and of significant changes in the liver.

Lesions Produced by Different Types of Pneumococci

Although different strains of the same type of pneumococcus differed in virulence when introduced intranasally into a standard group of mice, nevertheless the end result in those mice which died was found to be the same. Thus, it was found that, despite the varying mortality rate caused by different strains of the same type of pneumococcus, the lesions produced in those mice that died were closely similar for the one type of pneumococcus in a given breed of mice and were predictable. They differed, however, with the type of organism used. There had been few observations on this latter point.

The variation with the type of organism used is clearly brought out by the pathological changes found in those unselected albino mice which died as a result of the intranasal inoculation of different types of pneumococcus, as described elsewhere (1). Autopsies were performed on mice dying from the intranasal inoculation of Types I, II, III, and V strains. Three strains of Type I were used, four of Type II, two of Type III, and one of Type V. Twelve mice died from both Type I and Type II strains, eleven from Type III strains, and only one from the Type V strain. In every case in this experiment an autopsy was performed within 30 minutes of the time of death of the mouse, and no mice dying during the night were included. Since the lesions were very similar for the strains of one type but differed from type to type, the mice dying from infection with each type will be described together.

Type I and Type V.—The lesions in the single mouse dying from a Type V infection were so similar to those of the Type I group that they will be described with this group.

Macroscopically, the changes in the thoracic cavity were the most conspicuous. Pneumonic consolidation was infrequent, but the lungs were usually dark red and moist on section, unlike the normal pink, crepitant lung. Pleurisy was common and consisted usually of a thin layer of fibrin dulling the shiny surface of the lung. Effusion when present was scanty, thin, and watery. The bronchial and paratracheal nodes were normal. The cervical nodes were occasionally enlarged and opaque, but this was not a common finding. The spleen was also enlarged in several instances, but without macroscopic changes. In a certain number of cases petechiae could be seen on the surface of the kidneys.

Microscopically, one mouse out of the thirteen showed completely normal lungs, and in two others the lesions were very slight. The rest showed moderate or marked changes. In what appeared to be the earliest stages, the interalveolar

capillaries were dilated and filled with blood. There was often a slight pleural exudate of fibrin and polymorphonuclears, and the subjacent lymphatics would be filled with an exudate similar to that on the pleura. The blood vessels showed a dilatation of spaces in the region of, or more probably just outside, the adventitia which were presumably lymphatics. This dilatation increased in extent as the vessels ran centripetally, and the spaces contained serous fluid, fibrin, and pneumococci with but few cells. In this early stage there was very little exudate into the alveoli, and what was present consisted of fluid and pneumococci usually with a very few cells. The changes were scattered diffusely through both lungs. The blood vessels contained many pneumococci. In the later stages all changes became markedly increased. The vascular engorgement was extreme and the alveolar capillaries contained large numbers of leucocytes, both polymorphonuclears and monocytes. Pleural exudate when present was conspicuous and consisted of a layer, 1 mm. or more in thickness, of fibrin, polymorphonuclears, and organisms. The subpleural lymphatics were sharply outlined with their content of polymorphonuclears, fluid, and organisms, and this could be followed through the perivascular lymphatics to the hilum. The peribronchial lymphatics were very rarely involved. There was more exudate into the alveoli. In some places this was diffuse and consisted of serous fluid and pneumococci with relatively few cells, while in others it was found in small localized areas of a few alveoli and consisted of masses of leucocytes and a little fibrin tightly interwoven (Figs. 1, 2, 3). Pneumococci were not as plentiful in the blood stream as in the earlier stages.

The cervical nodes were enlarged, but rarely showed definite lesions. Occasionally there was an acute lymphangitis of the entering or leaving lymphatics which were dilated and filled with fluid, polymorphonuclears, and pneumococci. These nodes lie in close relation to the salivary glands and presumably drain the mucosa of the nasopharynx and throat. The spleen was also enlarged and occasionally showed necrosis of the Malpighian corpuscles. The normal structure was lost and the corpuscles were occupied by masses of polymorphonuclears, debris, and pneumococci. In one case there were several small infarcts in the liver, but otherwise this organ showed little but cloudy swelling or fat in the cells at the periphery of the lobule. The kidneys were the seat of a marked change which amounted in many cases to a diffuse nephritis. There was some proliferation of the endothelial cells of the glomerular capillaries, and in many cases the latter were occluded with fibrin thrombi (Fig. 12). The glomerular capsule spaces contained debris, and adhesions between tuft and capsule wall were found. In a few cases blood was found in the tubules that had come presumably from the glomerulus above. Besides blood, the dilated tubules contained loose casts of debris and, occasionally, pneumococci. The cells of the convoluted tubules frequently contained small hyaline droplets, described elsewhere (12) as colloid granules, and in a certain number of cases this lesion was striking. In all, ten out of the twelve mice showed changes which could be described as acute diffuse nephritis.

Type II.—Macroscopically, the most conspicuous changes were again in the thoracic cavity. Consolidation, which was often lobar in extent, was more commonly observed than in the other series, while the dark red, moist lung and the rather dry pleurisy were similar to those changes noted in the Type I group. It was very unusual to find any change in the lymph nodes whether tracheal, bronchial, or cervical, though in one case the latter were slightly enlarged and opaque. The spleen was not infrequently several times its normal size but showed no macroscopic lesions. The kidneys and other organs showed no changes.

Microscopically, in the ten of the twelve mice which showed lesions in the lung the peculiarity lay in the greater amount of exudate into the alveoli in the Type II infection than in those due to Type I or Type III infections. Even in the early stages the exudate was conspicuous, the alveoli containing large amounts of serous fluid and many pneumococci, together with a few cells of which the larger number were red cells. Other changes included capillary dilatation; early pleural effusion and exudate; dilatation of the subpleural lymphatics which were filled with fluid, cells, and organisms; and many organisms in the blood vessels. In the later stages, the alveolar exudate showed an increase in the number of leucocytes, and a slight deposition of fibrin (Figs. 4, 5). The organisms were present in the alveolar exudate but had decreased in the blood. The pleural exudate and involvement of the perivascular lymphatics were more marked.

No changes were noted in the cervical nodes. In the spleen the follicles occasionally showed areas of necrosis, at times so extensive as to obliterate the whole follicle. In one case, the portal veins of the liver contained thrombi and these had produced small cortical infarcts. The changes in the kidney were not as striking as with the Type I infection. The glomerular capsular spaces were dilated and contained albuminous fluid and pink hyaline debris. In two cases, the convoluted tubules contained small amounts of blood coming presumably from the glomerular tuft above. The most conspicuous changes were in the tubules. These were dilated, and the lining cells showed cloudy swelling and occasional necrosis with a loss of cytoplasm into the lumen of the tubule; special stains revealed colloid granules in the cytoplasm in many cases (Fig. 13). The tubules contained well formed casts of loose plugs of debris, the latter coming both from the glomerular transudate and from the cytoplasm of the cells of the tubules.

Type III.—Macroscopically, the picture of the infection due to Type III strains differed from those already described. The changes in the thoracic cavity were still very striking, but not in every case were they the most conspicuous lesion. Pleurisy with a copious gelatinous exudate was a very frequent finding. It was often associated with a mediastinitis and pericarditis, less frequently with a peritonitis. The change most commonly seen in the lungs was a large, moist, dark red lung; but consolidation, which tended to be lobular, was more common than in Type I cases. The other conspicuous change was in the lymphoid tissue. Bronchial and tracheal nodes were sometimes enlarged and opaque or even frankly

necrotic, but most striking were the changes in the cervical nodes which were usually enlarged and opaque, often necrotic and occasionally, the infection having spread to the surrounding tissues, were actually sloughing through the skin. In many cases the swollen lymph nodes obstructed the free return of lymph and the face region of the affected mice became swollen and edematous, especially on the side on which the nodes were principally affected (Fig. 15). The spleen was constantly enlarged, filled with blood, and showed opaque yellow nodules occupying the lymph follicles. In three cases, the enormously engorged spleen had ruptured and an extensive hemorrhage had occurred into the peritoneum. The other organs showed no macroscopic changes.

Microscopically, two of the mice showed no lesions in the lungs. In the other nine, the lesions differed from those due to either Type I or Type II strains. A most important feature was the tendency for the greater part of the lesion to be interstitial and localized in the alveolar septum. This, in the earliest stages, was shown by the very marked dilatation and engorgement of the alveolar capillaries (Fig. 6). At first, leucocytes were scanty, but later they increased in number. Diapedesis took place and large numbers of leucocytes together with fluid and some fibrin collected outside the vessels but within the septa (Figs. 7, 10). Later, there was a true interstitial inflammation progressing as far as necrosis of the septa. In two cases of the Type III infection, infiltration occurred round the main bronchi, a lesion never observed with the other types. The peribronchial lymph spaces were dilated and contained fluid, leucocytes, and a small amount of fibrin. Subpleural and perivascular lymphatic infiltration, similar to that described above, was also present. Alveolar exudate was similar to that found in Type I. In one case there was a plug of cells and debris in the bronchus, but in all cases the bronchial mucosa was intact.

Lesions were found in the lymph nodes at the hilum of the lung, in the cervical nodes, and in the follicles of the spleen. In the nodes, the change began as an enlargement of the node due to a swelling of the peripheral lymphatics which were filled with mononuclear cells and some organisms. Later, polymorphonuclears appeared and increased in number, while at the same time the pneumococci became more plentiful and areas of necrosis appeared. These increased in size until they filled the whole node, the normal structure of which was completely destroyed (Fig. 14). There could be found an acute lymphangitis of the entering or leaving lymphatics. The lesions in the cervical nodes were the most conspicuous and were often present when the nodes at the hilum of the lung were normal. In the follicles of the spleen, the lesion began in the center. Some of the lymphoid cells appeared necrotic and pneumococci could be seen in the neighborhood. Following this, there was a loss of most of the lymphoid cells, though whether chiefly from necrosis or from migration away from the site could not be determined, and only the framework of the follicle remained with polymorphonuclears lying at the periphery. Still later, these polymorphonuclears filled the follicle round a necrotic core, and a true abscess was formed (Fig. 16). In one case, small areas of necrosis were found in the liver, but otherwise there was nothing but cloudy swelling. The kidney showed nothing but cloudy swelling of the epithelium of the tubules.

In summarizing the lesions produced in a standard breed of mice by strains of pneumococci belonging to different types, the following points are most worthy of emphasis (Table I). As far as the lungs are concerned, the lesions differed considerably in the three types. In Type I, the changes were diffuse, without any particular point of localization and without lobar distribution. In the lungs from the Type II infections, the exudate into the alveoli and the lobar distribution were the most conspicuous features, while pleural exudate was infrequent. In the Type III cases, the localization of the lesion in the interstitial tissue of the alveolar septa was the most conspicuous difference, but there was also to be observed a very infrequent peribronchial infiltration. Copious gelatinous pleural exudate was also a feature of the Type III infections. In this group also were found the marked lesions in the lymphatic system, cervical nodes, hilar nodes, and lymph follicles of the spleen. Similar changes were found in the mice infected with the other types, but much more rarely and in lesser degree.

In the other organs the renal lesions were most striking. In ten out of twelve Type I cases there was an acute diffuse nephritis; in the Type II cases lesions were also found, but were predominantly tubular; while the kidneys of the Type III infections showed no lesions worthy of note. It is interesting that rabbits infected with Type I, Type II, and Type III strains of pneumococci showed renal lesions only with the Type I strains (12, 13), thus resembling the mice, but it must be stated that in the rabbits no attention was paid to the virulence of the strains of pneumococci used, and that most of them were stock strains.

The Development of the Pneumonia Following Intranasal Inoculation

In order to obtain information as to the origin and mode of spread of this pneumonia in mice following intranasal inoculation, serial sections of lungs were examined. It is realized, of course, that this method can merely suggest the point of origin and method of spread, and shows little or nothing as to the method by which the organisms reached the presumed point of origin. Serial sections were made only in Type III pneumonias.

As a result of the examination and a comparison of these findings with those obtained by the routine examination of several sections

from each lung, certain statements can be made. The earliest lesion in every case of Type III pneumonia, and the only one which is found by itself, is a dilatation and engorgement of the capillaries, especially those of the interalveolar septa (Fig. 6).

It is difficult to say which, if any, of the three changes next to be described precedes the others. These are: the collection of cells and fluid in the alveolar septa but outside the capillaries—an interstitial lesion (Figs. 7, 10); the exudate of albuminous fluid, rich in organisms but poor in cells, into the alveoli (Figs. 8, 11); and the dilatation of the subpleural and perivascular (but not the peribronchial) lymphatics with fluid, pneumococci, and monocytic cells.

The first two changes—the early exudate and the interalveolar inflammation—occur apparently independently in different parts of the lung or different parts of the same lobe. In a certain number of cases the interstitial lesion seems to precede the exudate and is well marked in the walls of alveoli in which exudate is just appearing. Yet, this is not always so, and often the walls of alveoli filled with exudate appear merely congested. It may be that in such cases the interstitial inflammation has been of ephemeral character and has already disappeared. The lymphatic involvement seems to follow a little later, and this is probably always the case with the subpleural lymphatics. It may appear in some microscopical preparations that lesions are present in the perivascular lymphatics in lobes with no other lesion save engorgement, but it has never proved possible to demonstrate this in serial section.

One can state then that in the mouse the pulmonary changes seem to progress in the following stages: (*a*) engorgement; (*b*) interalveolar interstitial exudate; (*c*) an albuminous fluid exudate into the alveoli and into the perivascular and subpleural lymphatics draining the affected region.

From now onwards, each separate process develops almost independently. The alveolar exudate becomes more cellular and eventually strands of fibrin, always less in amount than in pneumonia in man, are laid down. The interstitial inflammation may subside, but occasionally becomes so intense that the whole septum is destroyed and its structure lost. The subpleural and perivascular lymphatics become still more dilated and the contents become largely cellular

with polymorphonuclears predominating (Fig. 9). The pleura overlying the affected lymphatics is the site of an inflammatory exudate—an acute pleurisy—and this may be almost as early a lesion as the exudate into the alveoli.

The spread along the lymphatics can be followed to the nodes at the hilum. In these, the earliest change is one of dilated sinusoids which contain mononuclear cells and a few organisms. Later, these nodes become the seat of an intense inflammation so that the whole organ is converted into an abscess which ruptures and the organisms escape out into the mediastinum to produce an acute mediastinitis. The paratracheal nodes, lying higher up along the trachea, are always affected much later.

The cervical adenitis appears to have no direct connection with the pulmonary lesions as outlined here. In many cases—perhaps the majority—the most severe cervical adenitis is found in association with lungs that are almost normal or show little else than congestion.

SUMMARY

Pneumonia can be produced in mice, which have not been previously prepared, by intranasal inoculation of broth cultures of certain strains of pneumococci.

Lesions which are quantitatively different can be produced in different breeds of mice by inoculation of the same type of pneumococcus. Similar inoculation of different types of pneumococci into one breed of mice results in lesions which are qualitatively different.

In general, these lesions are as follows: a diffuse pneumonia and an acute glomerular nephritis in unselected mice receiving Type I strains; a confluent pneumonia and a tubular nephritis in the case of Type II strains; and as result of Type III strains, an interstitial pneumonia with extensive gelatinous pleurisy, together with necrosis and abscess formation in the spleen and cervical lymph nodes. Resistant strains of mice with Type III pneumococci show slight changes in the lungs, but marked lesions in the spleen and cervical nodes, while susceptible mice with the same type of pneumococcus show marked changes in the lung and moderate lesions in the spleen and cervical nodes.

The method of development of Type III pneumonia, studied by means of serial sections of nasally infected mice, appears to proceed

in the stages of vascular engorgement, interalveolar interstitial exudate, albuminous fluid exudate into the alveoli and the perivascular lymphatics draining the affected site, and finally, a frank pneumonia with a cellular exudate in the alveoli but without much fibrin.

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EXPLANATION OF PLATES

Plate 3

Fig. 1. Type I pneumonia. Localized area of leucocytic exudate into alveoli just below pleura. There is a thin film of pleural exudate on the surface. Eosin-methylene blue. ×100.

Fig. 2. Type I pneumonia. Similar localized pneumonic area in the substance of the lung. At the lower right corner a portion of a blood vessel appears with a dilated lymphatic channel surrounding it, which is filled with leucocytes. Eosin-methylene blue. ×100.

Fig. 3. Type I pneumonia. Higher magnification showing the type of cells forming the alveolar exudate. Eosin-methylene blue. ×650.

Fig. 4. Type II pneumonia. Confluent area of pneumonia. The alveoli contain masses of leucocytes and necrotic debris. The lymphatic channels around the two larger vessels are greatly dilated and filled with leucocytes and debris. Eosin-methylene blue. ×100.

Fig. 5. Type II pneumonia. Higher magnification showing the alveolar exudate of leucocytes and debris. Eosin-methylene blue. ×650.

Plate 4

Fig. 6. Type III pneumonia. Showing the general vascular engorgement. Eosin-methylene blue. ×100.

FIG. 7. Type III pneumonia. Great thickening of the alveolar walls with the collection of leucocytes inside and outside the alveolar capillaries. Eosin-methylene blue. ×100.

FIG. 8. Type III pneumonia. Showing many alveoli filled with albuminous fluid. Eosin-methylene blue. ×100.

FIG. 9. Type III pneumonia. Most of the alveoli are filled with a mass of leucocytes, debris, and fibrin. Many alveolar walls are necrotic. In the lower part of the field is a blood vessel with an enormously distended lymphatic channel filled with fibrin and leucocytes. Hematoxylin-eosin. ×100.

FIG. 10. Type III pneumonia. High magnification showing the interstitial infiltration of the alveolar walls. Eosin-methylene blue. ×650.

FIG. 11. Type III pneumonia. High magnification showing the alveoli filled with albuminous fluid and a few cells. There is interstitial infiltration of the walls. Eosin-methylene blue. ×650.

PLATE 5

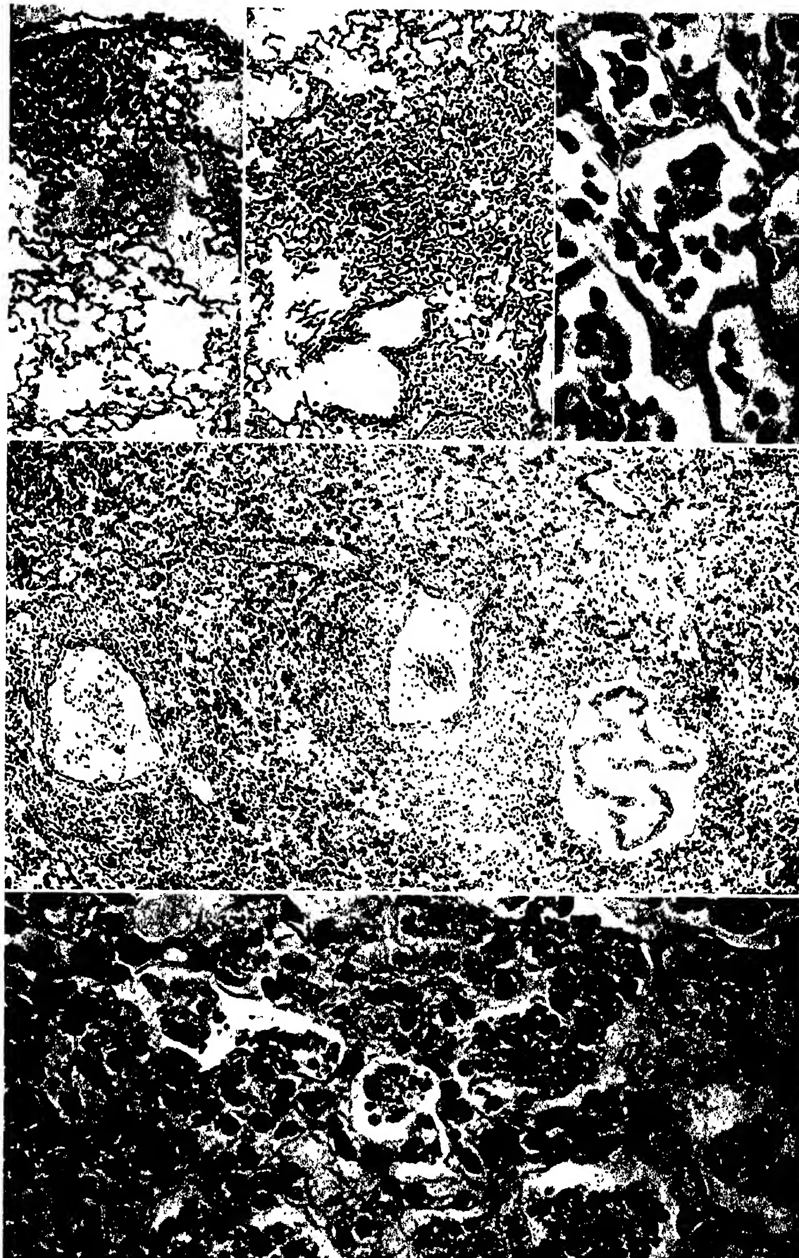
FIG. 12. Type I infection Renal glomerulus with capillary loops occluded with fibrin thrombi. Weigert's fibrin stain. ×650.

FIG. 13. Type II infection. Deeply staining "colloid" granules in the cells of the convoluted tubules. Weigert's fibrin stain. ×650.

FIG. 14. Type III infection. Cervical lymph node the center of which is occupied by an abscess. The main lymph vessel to the right also shows inflammation. Portions of the salivary glands may be seen. Eosin-methylene blue. ×100.

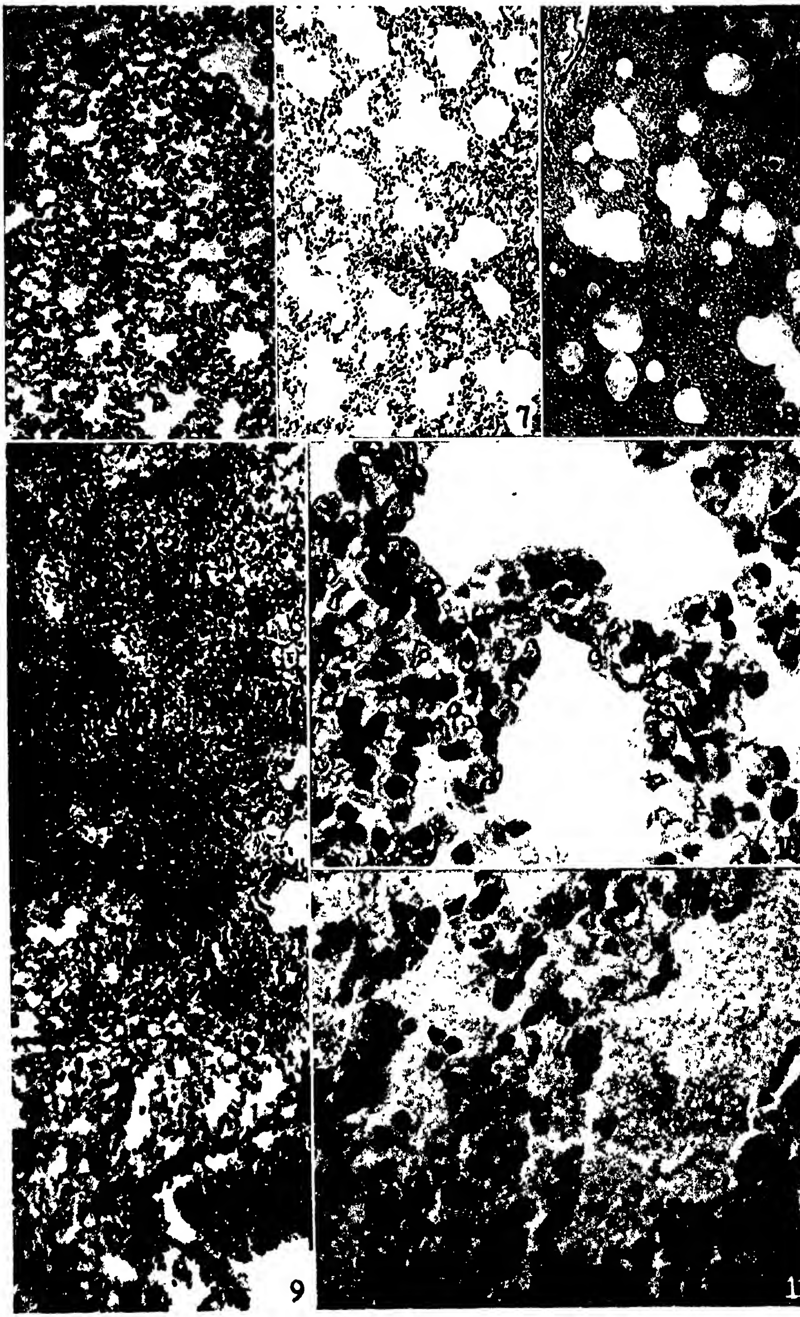
FIG. 15. Type III infection. Albino mouse showing ulceration through the skin below the left mandible (three dark areas through the hair), and great swelling of the whole head and face from edema. The eye is closed by the edema. Natural size.

FIG. 16. Type III infection. Spleen showing outline of Malpighian corpuscle the center of which is occupied by an abscess. Eosin-methylene blue. ×100.



Photographed by Louis Schmidt

(Rake: Pneumococcus infection in mice)



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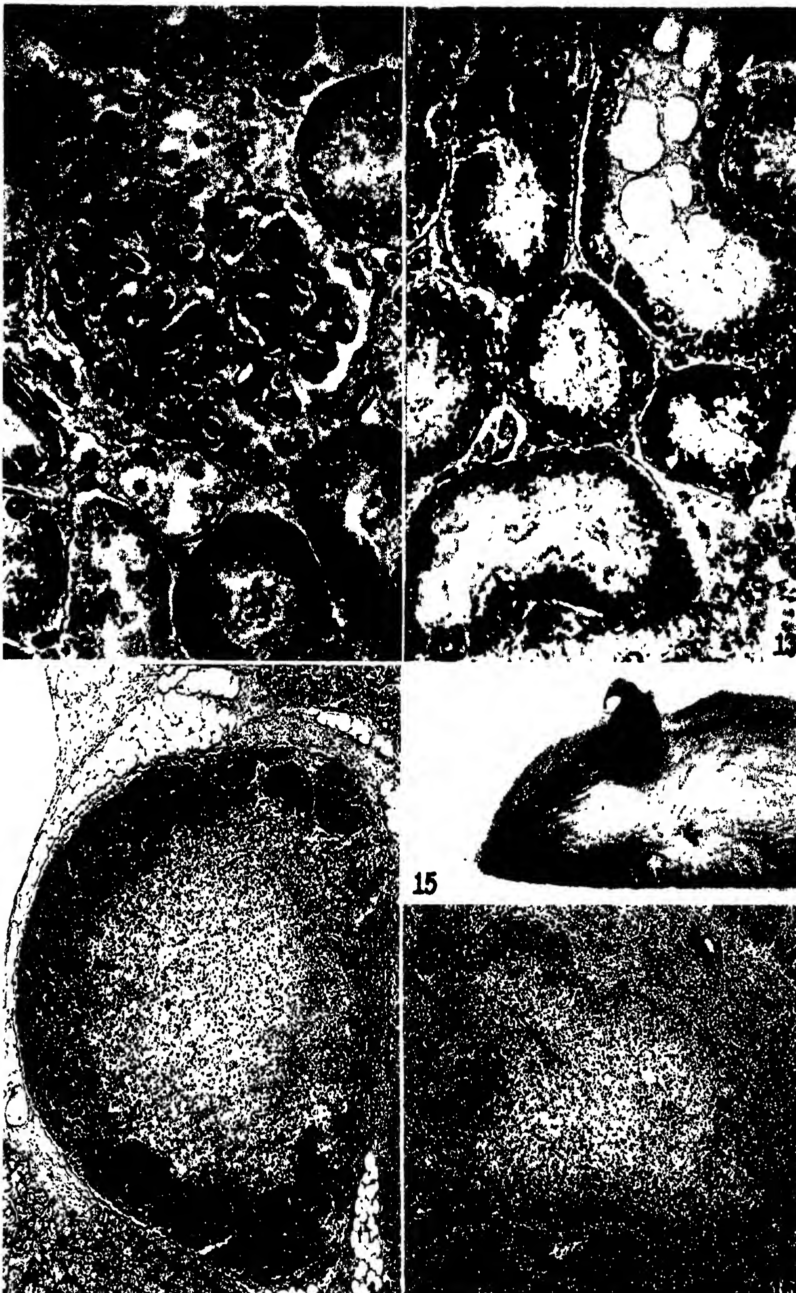
(Rake: Pneumococcus infection in mice)



Photographed by Louis Schmidt

(Rake: Pneumococcus infection in mice)

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EXPERIMENTS ON ACTIVE IMMUNIZATION AGAINST EXPERIMENTAL POLIOMYELITIS

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The results of many efforts to induce resistance in *Macacus rhesus* monkeys against experimental poliomyelitis by means of inoculations of virus in one form or another have been summarized by Stewart and Rhoads (1929) (1) and in the volume published by the Milbank International Committee (1932) (2). What may be derived from these experiments, beginning with the first, undertaken 25 years ago by Flexner and Lewis (3), is that "it is impossible to protect monkeys by the use of killed virus and second, that a definite though inconstant resistance to poliomyelitis can be brought about by the intradermal and subcutaneous introduction of the living virus" (1). The fact also emerges from the numerous trials hitherto reported that resistance is acquired by monkeys when a sufficient amount of active virus is given intra- or subcutaneously in one massive dose (3, 4) or in smaller amounts repeated over a considerable period of time (3–6). Even then protection is not afforded to some animals and the degree of immunity induced varies in others, while now and again a treated monkey succumbs to the disease as a result of the inoculations (1, 5–8).

Two noteworthy series of articles have recently appeared, in one of which was described the immunity obtained through the use of virus completely inactivated by 0.1 per cent formalin (Brodie, 9) and in the other the protection conferred with active but ricinoleated virus (Kolmer and his associates, 10). While the principles underlying both methods had already been employed (2), the recent investigators report results which lead them to believe that immunity can be safely induced with their materials.

Since on the basis of Brodie's and Kolmer's work widespread inoculations of children against poliomyelitis have been undertaken recently,

it was deemed desirable to restudy this problem, following as closely as possible the methods of these investigators. The intention was to determine whether any advance has been made in the experimental immunization of monkeys over that which has been accomplished in the past 25 years, and whether any procedure has been disclosed that might be practical for immunization of man.

For the purpose of comparison we also studied another form of chemically treated virus as immunizing agent, namely, that precipitated by tannin, which will be described first.

Methods

Virus.—The animals selected as source of poliomyelitis virus were extensively paralyzed and moribund as a result of the experimental disease. They were killed by ether inhalation and the spinal cord removed under aseptic conditions. The identification of the particular virus used in the preparation of each immunizing agent was ascertained by (*a*) animal inoculation with production of specific clinical signs and pathological changes, and by (*b*) neutralization with specific homologous strain antiserum. The M.V. and Philadelphia strains (11) of virus were employed.

Method of Testing for Acquired Active Immunity.—Monkeys were tested for induced resistance by the inoculation of homologous strains intracerebrally and intranasally.

The intracerebral test dose[1] consisted of 0.2 cc. of 5 per cent fresh poliomyelitis cord suspension which was filtered through a Berkefeld N filter. Kolmer (10), working with the M.V. strain, states that one infective unit was contained in 0.05 cc. of an unfiltered suspension in some instances and in 0.2 cc. in others. However this may be, the high cost of monkeys makes it impractical to titrate each individual virus sample; hence the test dose for induced resistance should be one that experience has shown to be unequivocally effective. The dosage as given in the following experiments has been consistently employed in this laboratory for many years with satisfactory results. Normal monkeys receiving it react with the experimental disease within, as a rule, 5 to 11 days; only exceptionally does an animal resist. All the controls of the following series of tests developed the characteristic infection.

The intranasal test for induced immunity consisted of the instillation into each nasal cavity of 1 cc. of 10 per cent glycerolated cord suspension, and after 1 or 2 days' interval the treatment was repeated. The reaction was measured not only by clinical signs but also by cell counts of the spinal fluid withdrawn daily through cisternal puncture. The method is essentially that of Flexner (12) and his associates and suffices satisfactorily to determine the state of immunity in a treated

[1] All such inoculations were made with the aid of full ether anesthesia.

animal. It may be said that the amount as given is not too drastic since in a collateral series of twenty-four monkeys, twenty-one developed poliomyelitis; the three unaffected ones could not be considered immune, only uninfluenced by the treatment, since one of them—the only one retested—was later shown to be susceptible to a similar intranasal instillation of virus. Hence the test dose as practised is in the range of minimal infective dosage. It is of interest that all controls so treated which were employed in the experiments to be reported were successfully infected.

Test for Antiviral Bodies in Serum.—0.8 cc. of undiluted serum is mixed with 0.2 cc. of 5 per cent filtered fresh cord virus, kept at 37°C. for 2 hours and in the cold for 16 to 18 hours, and then injected into the brain of monkeys. For control, the serum is replaced by physiological saline solution. Here again neutralization tests are carried out with the homologous strain of virus. The test can be regarded as a practical one even though the precise titration of antibody content of a serum is not ascertained.

Tannin-Precipitated Virus as Immunizing Agent

In a correlated study on the virus of equine encephalomyelitis, it was found that vegetable-derived tannin (tannic acid) precipitated the proteins of the tissue containing the virus and the latter precipitated with the proteins remained infective although somewhat reduced in potency (13). The virus could not be designated as "attenuated" but merely as present in lesser amounts in the flocculated substance. Under these conditions the infective agent retained its activity for several weeks. As the following will show, similar results were obtained with tannin precipitates of active poliomyelitis tissues.

Preparation of Immunizing Agent.—2.5 gm. of poliomyelitis cord were thoroughly ground with sand and suspended during the grinding in 50 cc. of distilled water. The suspension was spun in an angle centrifuge for 15 minutes at 2,000 R.P.M. The supernatant fluid only was retained and was decanted into a 100 cc. centrifuge flask and 5 cc. of 2 per cent aqueous solution of Mallinckrodt's tannic acid were added. The mixture was energetically shaken and then stored overnight in the cold. After about 18 hours the material was again shaken and centrifuged for 20 minutes at 3,000 R.P.M. The supernatant fluid was discarded and the precipitate washed, with stirring, in 50 cc. of Tyrode's solution. After similar centrifugation, the sediment was collected and resuspended in 50 cc. of hormone broth, pH = 7.6. This suspension was stored in the cold and used as immunizing agent from 3 to 14 days after its preparation.

The tannin-precipitated virus was injected subcutaneously in the amounts to be mentioned and in several instances produced locally small, indurated masses which regressed after 1 or 2 weeks.

TABLE I

Tannin-Precipitated Virus as Immunizing Agent

Monkey No.	Immunization			Tests for active immunity					Tests for passive immunity	
	Antigen	Amount given	Result	Route	Dose	Result	When retested with Phila. virus nasally (2 doses)	Result	Serum procured after last I.D.	Result
									days	
1	M.V. virus 5% fresh cord + tannin	2 cc. S.C. 3 times at 7–11 d. intervals	Died of enteritis 2 d. after 3rd I.D.							
2	" "	" "	Died of Tb. 15 d. after 3rd I.D.							
3	" "	" "	N.S.	I.N.	2 doses M.V. virus 71 d. after 3rd I.D. (3 controls, all P. 6 d.)	P. 5 d. sp.fl. 28–565			10	Pooled serum neutralized (control, P. 8 d.)
4	" "	" "	"	"		P. 6 d. sp.fl. 32–740			10	
5 (control)	" "	0.5 cc. injected I.C.	"							
6	Same with Phila. virus	2 cc. S.C. once	P. 8 d.							
7	" "	" "	P. 10 d.							

8	" "	2 cc. S.C. 3 times at 14–8 d. intervals	N.S.	I.N.	2 doses Phila. virus 41 d. after 3rd I.D. (control, P. 8 d.)	N.S. sp.fl. 21–45	260 d. after last test (2 controls, P. 6 and 9 d.)	N.S. sp.fl. 15–27 cells	15	Serum neutralized (control, P. 8 d.)
9	" "	" "	"	I.C.	Phila. virus 51 d. after 3rd I.D. (control, P. 5 d.)	P. 6 d.			15	" "
10 (control)	" "	1 cc. injected I.C.	P. 6 d.							
11	Phila. virus 1% glycerolated cord + tannin	1,1,2 cc. S.C. at 9–10 d. intervals	N.S.	I.N.	2 doses Phila. virus 22 d. after 3rd I.D. (control, P. 8 d.)	No paralysis. Sp. fl. 14–710 (see text)	260 d. after last test (2 controls, P. 6 and 9 d.)	P. 8 d. sp.fl. 17–310 cells	15	Pooled serum neutralized (control, P. 8 d.)
12	" "	" "	"	"		No paralysis. Sp. fl. 27–105 (see text)	" "	P. 6 d. sp.fl. 25–974 cells	15	
13	" "	" "	"	I.C.	Phila. virus 32 d. after 3rd I.D. (control, P. 5 d.)	P. 6 d.			15	Pooled serum failed to neutralize (control, P. 8 d.)
14	" "	" "	"	"		P. 5 d.			15	
15 (control)	" "	1 cc. injected I.C.	"							

I.D., immunizing dose; S.C., subcutaneous injection; d., day; Tb., generalized tuberculosis; N.S., no symptoms; I.N., intranasal; P., characteristic experimental poliomyelitis, the number following showing the day on which paralysis was first observed; sp.fl., pleocytosis, the first figure representing normal cell count and the second, the reactive count; I.C., intracerebral injection; Phila., Philadelphia 1932 strain of virus.

Results of Preventive Inoculations.—It will be seen from Table I that of eight monkeys injected subcutaneously with the 5 per cent virus suspension (M. V. and Philadelphia strains), two died of non-poliomyelitis affections and of the remaining six, two became moribund after an attack of poliomyelitis following the first subcutaneous dose of 2 cc. In the one instance in which the antigen was Philadelphia virus, 1 cc. inoculated intracranially induced poliomyelitis in a control monkey. When the immunizing agent was reduced in content of virus to 1 per cent of cord by weight and only a total of 4 cc. of it was given subcutaneously to each of four monkeys and 1 cc. intracerebrally to a fifth, none of the five so treated developed disease.

The data in Table I clearly show that material containing active virus can by itself give rise to fatal infection after a single subcutaneous injection. It is significant, however, that of two monkeys receiving three such doses of the same virus sample, one failed to be protected against a subsequent intracerebral test inoculation but the second resisted the intranasal test instillation. Results based on the reactions of only two animals are inconclusive but they serve to bring out one of the difficulties met with in attempting to immunize animals with active virus preparations.

The power of the various tannin-precipitated virus preparations to build up resistance was not great, for it is noted that of three monkeys receiving the Philadelphia virus and which were given the intracerebral test inoculation, all developed poliomyelitis. Of the treated animals injected with the 5 per cent antigen and tested intranasally for immunity, two succumbed and one monkey was found to be resistant to this and a repeated test. Of those receiving the 1 per cent material, both were resistant to the first intranasal test dose but were susceptible to a second test.

Hence only one of the five animals receiving the immunizing agent was found resistant to the intranasal test and that one resisted both of two tests. Even so, one cannot regard this monkey as immune, for, as Flexner (14) shows, monkeys can be refractory to several successive courses of instillations yet respond to a final one of the same virus. In addition, these results confirm Flexner's finding that when virus is placed in contact with the nasal mucosa, pleocytosis may occur, but the increase in the number of cells in the spinal fluid may not be

associated with symptoms of infection or with the development of immunity.

The capacity of tannin-precipitated virus to produce serum antiviral bodies is varied. Of the series injected with 5 per cent virus antigen, the pooled serum of two monkeys and individual sera of two others neutralized virus by the method described; of the animals given the 1 per cent antigen, the pooled serum of two neutralized and that of two others failed to do so. To be noted is that in five instances treated monkeys yielded neutralizing serum but were found, 51, 71, and 301 days after the last immunizing dose was given, to be susceptible in average degree to intracranial or intranasal contact with virus. This is not unusual; it has recurred in the experiments soon to be described with formalin and ricinoleate. Moreover, Stewart and Rhoads (1), Schultz and Gebhardt (15), and recently Aycock[2] and others have reported the lack of correlation existing between serum antiviral bodies and immunity as tested by the cerebral or nasal routes. In other words, the presence in the monkey of serum antiviral bodies, as produced by artificial immunization and determined by the described method, is no definite indicator of the state of active resistance of the animal to the test doses used.

To summarize the results of preventive treatment with tannin-precipitated poliomyelitis virus, it would appear that this product has failed as a satisfactory immunizing agent and that it is restricted by the same uncertainty which living virus as such manifests as a preventive when injected under similar conditions. Too much of the material can induce infection; too little, inconstant and unreliable immunity.

Active Ricinoleate-Treated Virus as Immunizing Agent

Kolmer (10), basing his experiments on those of McKinley and Larson (16), employed 1 per cent sodium ricinoleate to attenuate but not inactivate the poliomyelitis virus in 4 per cent cord suspensions prepared from 1 month old glycerolated tissue. The ricinoleated material was kept in the cold for 1 month before use and then in one series of experiments 0.1 cc. of the agent per kilo body weight was injected subcutaneously five times at 5 day intervals into seven monkeys, and similar dosages were given intracutaneously to three additional animals. They

[2] Personal communication.

showed no symptoms, and 1 month after the last treatment, when subjected to an intracerebral inoculation of 0.2 cc. of 5 per cent virus suspension, one developed poliomyelitis and the others were unaffected. The survivors were again injected intracerebrally with virus up to 17 months later and all but one survived a third cerebral test for resistance.

In repeating the experiment with sodium ricinoleate-treated virus, we used the same virus (M.V. strain) which Kolmer employed and the sodium ricinoleate was sent us through the kindness of the same manufacturers.[3] The methods were those of Kolmer except as regards the intracerebral test dosage: Kolmer employs as a test dose for induced immunity unfiltered and we, filtered suspensions. In addition, we employed nasal instillation, as described, for this purpose, a procedure which he omitted.

Results of Preventive Inoculations with Ricinoleate-Treated Virus.—Reference to Table II shows that six monkeys received the Kolmer vaccine. Of two tested intracerebrally for immunity, both failed to resist and of four instilled intranasally, two developed the disease on the first instillation and a third on a repeated test. Thus only one of the six animals resisted the tests for acquired resistance.

Table II reveals that the pooled serum of two treated animals and the individual sera of the remaining four neutralized virus in each instance. Here again, as occurred with tannin-precipitated virus, the antiviral bodies, as determined by the method given, were present but despite this fact the animals succumbed to the tests for active immunity.

When these experiments were well advanced, a paper was published by Schultz and Gebhardt (15), which stated: "The serums of another series of animals 'immunized' earlier with *living* virus (Kolmer vaccine) neutralized 30 M. I. D. doses of virus per cubic centimeter, but when these animals were subjected to intranasal instillation with active virus, they all developed typical poliomyelitis." We can thus confirm the findings of these investigators.

Formolized Virus as Immunizing Agent

In preparing materials, the methods of Brodie (9) were followed. 0.2 per cent formalin was added to 20 per cent active cord suspensions in equal volumes so that in the end 0.1 per cent formalin was in contact with 10 per cent virus suspension. This was kept at 37°C. for 16 hours, since at the time when this work was

[3] William S. Merrell and Co., Cincinnati.

TABLE II

Ricinoleate-Treated Virus as Immunizing Agent, Employing 4 Per Cent Glycerolated Poliomyelitis Cord Suspension + 1 Per Cent Sodium Ricinoleate, Subcutaneously

Monkey No.	Immunization		Tests for active immunity					Tests for passive immunity	
	Amount* M. V. virus antigen. 5 doses, 5 d. intervals	Result	Route	Dose given about 30 d. after last I. D.	Result	When retested with M.V. strain nasally (2 doses)	Result	Serum procured after last I.D.	Result
								days	
16	0.3 cc. (total 1.5 cc.)	N.S.	I.C.	M.V. virus	P. 6 d.			20	Pooled serum neutralized; (control, P. 6 d.)
17	0.27 cc. (total 1.35 cc.)	"	"	" "	P. 7 d.			20	
18 (control)	None	—	"	" "	P. 4 d.				
19	0.3 cc. (total 1.5 cc.)	N.S.	I.N.	M.V. virus 2 doses	N.S.	67 d. after last test	P. 11 d. (4 controls P. 7, 9, 10, 11 d.)	20	Serum neutralized; (control, P. 6 d.)
20	0.35 cc. (total 1.75 cc.)	"	"	" "	P. 8 d. sp.fl. 29–644 cells			20	Serum neutralized; (control, P. 6 d.)
21	" "	"	"	" "	P. 8 d. sp.fl. 45–730 cells			17	Serum neutralized; (3 controls, 2 P. 6 d. and 1, 22 d.)
22	0.34 cc. (total 1.7 cc.)	"	"	" "	N.S.	67 d. after last test	N.S. (4 controls same as Monkey 19)	17	Serum neutralized; (2 controls, P. 14 d. and 3 d.)
23 (control)	None	—	"	" "	P. 7 d. sp.fl. 330 cells				
24 (control)	1 cc. injected I.C.	P. 9 d.							

Abbreviations same as in Table I.

*0.1 cc. ricinoleate-treated virus per kilo.

TABLE III

Formolized Virus as Immunizing Agent,
Employing 0.1 Per Cent Formalin Fresh 10 Per Cent Virus (Cord) Suspension, Kept for 16 Hours at 37°C., Intradermally

Monkey No.	Immunization		Tests for active immunity					Tests for passive immunity	
	Amount given (2 x 5 cc. at 13 d. intervals) Strain	Result	Route	Dose	Result	When re-tested intra-cerebrally	Result	Serum pro-cured after last I.D.	Result
								days	
25	Phila.	N.S.*	I.N.	Phila. virus 2 doses 30 d. after last I.D.	P. 8 d. sp. fl. 24–834 cells			21	Pooled serum. Monkey febrile 7 to 11th d. T = 104.8°F.† Excited. Recovery (partial neutralization?); (2 controls, both P. 8 d.)
26	"	"	"	" "	P. 9 d. sp.fl. 17–224 cells			21	
27 (control)	None	—	"	Phila. virus 2 doses	P. 8 d. sp.fl. 22–420 cells				
28	Phila.	N.S.	I.C.	Phila. virus 36 d. after last I.D.	N.S.	57 d. after 1st test dose	N.S.	21	Pooled serum. Monkey febrile 7th to 13th d. T = 105°F., slight ptosis. Recovery (partial neutralization?); (2 controls, P. 8 d.)
29	"	"	"	" "	P. 6 d.			21	
30 (control)	None	—	"	Phila. virus	P. 7 d.				
31 (control)	"	—	"	" "	P. 7 d.				
32 (control)	2 cc. of formolized Phila. virus I.C.	N.S.							

33	M.V.	"	I.N.	M.V. virus 2 doses 30 d. after last I.D.	P. 8 d. sp.fl. 27–460 cells			20	Pooled serum. No neutralization; (control, P. 6 d.)
34	"	"	"	" "	P. 10 d. sp.fl. 33–340 cells			20	
35 (control)	None	—	"	M.V. virus 2 doses	P. 8 d. sp.fl. 25–514 cells				
36	M.V.	N.S.	I.C.	M. V. virus 50 d. after last I.D.	P. 8 d.			20	Pooled serum neutralized; (control, P. 10 d.)
37	"	"	"	" "	P. 5 d.			20	
38 (control)	None	—	"	M.V. virus	P. 8 d.				
39 (control)	"	—	"	" "	P. 7 d.				
40 (control)	2 cc. of formolized M.V. virus I.C.	N.S.*							

Abbreviations same as in Table I.

*The monkeys injected with formolized virus showed local skin necrosis with ultimate healing within about 2 weeks.

†T = highest temperature reading during febrile course.

done, Brodie stated that 12 to 16 hours of such contact served to inactivate poliomyelitis virus, and that the 16 hour material was employed by him as immunizing agent.

With respect to dosage for immunization of monkeys, it was first stated by Brodie (17) that one dose of 5 cc. yielded as good results as two doses of 5 cc.; this was later (18) changed so that it was then declared that two injections were more efficacious than a single intradermal one of 5 cc. In the following experiments, however, two doses of 5 cc. each were used throughout.

The intracerebral test for induced resistance as employed by Brodie was made with amounts on the borderline of infectivity, designated as "minimal completely paralyzing doses." In Table III, the intracerebral test was the same as given in the foregoing series of experiments with tannin and sodium ricinoleate, so that a proper comparison could be made of the different methods of immunization. This consisted of 0.2 cc. of filtered 5 per cent fresh cord suspensions. No mention is made by Brodie of determining immunity by means of intranasal instillation of virus; this we have carried out along with the intracerebral test.

Results of Preventive Inoculation with Formolized Virus.—As will be seen in Table III, of eight monkeys injected with formolized virus, only one resisted, and that one was found refractory to two successive intracranial test inoculations. It is common experience among workers in this field to meet with an occasional monkey refractory to poliomyelitis virus, so that it is uncertain whether the animal in question was immunized by the formolized material or not.

Of four sets of pooled serum, as indicated in Table III, one showed neutralization, another, none, and a third and fourth so called incomplete neutralization, due perhaps to low antibody content. The lack of correlation between serum antibodies with active protection has already been commented upon.

It is therefore plain that this method offers, under the experimental conditions employed, an ineffective immunizing material against poliomyelitis in monkeys.

The experience of Schultz and Gebhardt (15) employing the same agent is as follows:

They injected fifteen monkeys: three subcutaneously, four intramuscularly, and four intradermally, giving 0.1 cc. per kilo of 0.1 per cent formolized 10 per cent virus, and four intravenously with ten times this amount, five times at weekly intervals. 24 days after the last immunizing dose the animals received three M.I.D. of virus. "All developed the disease in about the same length of time, and with about as extensive paralysis as the controls, despite the fact that their serums seem

to have acquired slight, but definite virucidal properties." In additional experiments, Schultz and Gebhardt (15) injected the immunizing agent repeatedly in the brain of four monkeys and instilled it repeatedly in the nasal cavities of four others. All eight were proved susceptible to later inoculation with virus, in the brain in the first series and in the nose in the second.

The results we have obtained are corroborative of those of Schultz and Gebhardt, although the latter investigators employed a lesser amount of vaccine, and lead to the conclusion that formolized virus is not an effective preventive against poliomyelitis in the monkey. Other earlier observers (Abramson and Gerber, 19; Römer, 20; and Jungeblut and Engle, 21) also did not succeed in inducing immunity by means of formolized poliomyelitis virus.

DISCUSSION

The object of this study was the investigation of the problem of active immunization of *Macacus rhesus* monkeys by means of chemically treated poliomyelitis virus. The materials employed were tannin-precipitated virus and virus treated with sodium ricinoleate and with formalin. The latter two substances are those with which the vaccines of Kolmer and Brodie respectively are prepared and the tannin material introduced by us was employed for comparative observations.

The virus of poliomyelitis treated with tannin or sodium ricinoleate retains its activity so that intracerebral inoculation of monkeys with the preparations induces characteristic experimental poliomyelitis. Indeed, Kolmer (10) records that 0.2 cc. of his vaccine kept for 5 months when so inoculated induced the disease within 12 days. Further, the tannin-precipitated virus itself brought on infection in two animals after a single subcutaneous injection of 2 cc. It is therefore plain that the chemical treatment in both instances did not act to attenuate the virus.

The results of the experiments can be summarized by stating that if the immunizing agent contains a sufficient amount of virus, the danger arises of infecting an animal with the material itself. Under the experimental conditions employed, these preparations, although active virus was present in them, failed to immunize the inoculated animals regularly. Serum antiviral bodies were, however, produced

by means of the described methods but it was shown that animals in which these antibodies were present did not resist the ordinary tests for active immunity.

From what is here reported, it is apparent that there is no advantage to be derived from the use of the tannin-precipitated, or ricinoleated virus as immunizing agents over unchanged active virus, as employed in the past in this laboratory (Flexner and Lewis, 3; Flexner and Amoss, 22; Stewart and Rhoads, 1; and Rhoads, 23) and elsewhere (Aycock and Kagan, 5, and others).

A study of the recorded experiments of the past 25 years on immunization of monkeys reveals that active poliomyelitis virus itself is not a potent antigen, as are some other viruses; uniform protection is rarely brought about through its use. A greater degree of success in protecting animals can, however, be achieved when large doses over long periods of time are employed—which fact might lead one to suppose that the difficulty with poliomyelitis virus as immunizing agent may be related simply to the amount of antigenic substance present. Some viruses, such as those of equine encephalomyelitis (24) and yellow fever[4] among several others, can be diluted to 10^{-8} and still be infective for the most susceptible host, whereas poliomyelitis virus can be diluted to only a fraction of this amount to reach the limit of infectivity in the monkey. It is still unknown why the antigenic capacity of this virus is relatively less than that of several others. Finally, if amounts of virus sufficing to produce disease in some monkeys but not in others confer no immunity on the unaffected ones, it is to be expected that a lesser amount would be even less effective.

We now come to an estimation of formolized virus. In this instance, the evidence of earlier observers (2), later of Brodie (9, 17), of Schultz and Gebhardt (15), and ourselves points to the inactivation of the virus by the chemical. It is still an open question whether any form of inactive poliomyelitis virus retains the property of immunizing animals (2). An analysis of the results of the present investigation shows that active immunization with formolized virus by the Brodie method does not build up resistance in monkeys to the usual intracerebral or intranasal tests for induced immunity. The amount of antiviral bodies produced in the serum by this vaccine is slight and, as already indi-

[4] Theiler, M., personal communication.

cated, the treated monkeys failed, notwithstanding the presence of antibodies, to resist the tests for active immunity.

There are, therefore, discrepancies in the conclusions of Brodie (9) and ourselves. These may perhaps be ascribed to the fact that Brodie employs borderline dosages in his tests. With such small doses, it is possible that certain monkeys may not receive what for them is an infective dose of virus. At this point we wish to emphasize the fact that the intranasal test dose for immunity employed here was within the range of a minimal infective dose, as we have pointed out before; nevertheless, animals receiving formolized virus (or tannin-precipitated or ricinoleated virus) and among them even those which possessed serum antiviral bodies were found to be susceptible to this test.[5]

There remain for consideration the factors derived from animal experimentation which either Kolmer or Brodie maintains as a basis for the claims that a safe and successful immunizing agent has been made available for use in man.

The first factor which Kolmer (10) stresses as the essential one is the non-infectivity of his preparation. Kolmer admits that the degree of attenuation by sodium ricinoleate is slight or of minor importance but safety is acquired through the use of remote monkey passage virus that has apparently lost its infectivity for man. There is no experimental evidence for this assumption (25, 26).

The second is that ricinoleated and formolized vaccines engender in monkeys serum antiviral bodies and that the same mechanism might apply in man. It has been shown by Schultz and Gebhardt (15) and

[5] As this article goes to press, Brodie states (*J. Am. Med. Assn.*, 1935, **105**, 1089) that virus suspensions should be "just inactivated, for overtreatment or prolonged treatment with solutions of formaldehyde reduced the antigenicity of the vaccine," and therefore recommends the use of virus inactivated for 8 to 12 hours instead of 16. The distinction between "just inactivated" and "overinactivation" is not clear. In view of the still more recent modification (18) of 5 to 6 hours' contact with 0.1 per cent formalin at 37°C., it is apparent that this vaccine contains active virus as shown by Brodie in experiments in which 6 hours' treatment fails to inactivate the virus. The amount of active material may be small since, as Brodie points out, monkeys develop the disease only after repeated inoculation of 6 hour treated suspensions. It is known, however, that such small amounts of active virus do not induce protection in monkeys; still the possibility of infection during the period of immunization with an agent that contains active virus is ever present.

by us that the antibody response in monkeys is slight, although the Kolmer vaccine exceeded the Brodie preparation in this capacity. Despite the presence of acquired antiviral bodies in the serum no active resistance was developed to the recorded test doses for induced immunity.

Finally, the third factor relates to the active protection conferred on monkeys by means of the chemically treated virus. Since unchanged poliomyelitis virus lacks high antigenicity, it is to be expected that vaccines containing a lesser amount of active virus or virus that is inactivated would be still weaker in antigenic power. The results of the present experimental study reveal the ineffective and irregular immunizing capacity of these chemically treated viruses.

CONCLUSION

The results obtained in this investigation indicate that poliomyelitis virus treated with tannin, sodium ricinoleate, or formalin does not constitute a satisfactory immunizing agent in monkeys against the experimental disease.

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DIRECTION OF LIGHT, THE DOMINANT FACTOR IN THE PHOTOGRAPHY OF BACTERIAL COLONIES*

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(From the Division of Illustration of The Rockefeller Institute for Medical Research)

In books, magazines, and similar publications, it is usual to publish with the title the name of the author and illustrator, even when the latter is a photographer. In scientific publications, books as well as periodicals, the name of the artist illustrator usually appears on the reproduction of his drawing, but the photographer illustrator is very seldom credited for his share in the work. We should endeavor to have the name of the photographer printed on the pages carrying reproductions of his work. This would be a gratifying recognition of the photographer's contribution and would only be important to him. The fact that there is so little information printed with the photographs to describe the technique employed in their production is greatly to the disadvantage of the author and the reader.

In the description of plates the information is far too meagre. With photomicrographs a statement of the magnification usually suffices and generally this is given; less frequently the stain used in the preparation is also stated. A statement concerning the lenses used would help the reader if he desired to make similar photographs, and some readers have a clearer understanding of the optical picture if they know the lenses employed than if they know the magnification which is usually stated in figures, i.e., $\times$ 100.

There are classes of biological photography where a complete description of the technique employed is of real scientific value. An example is the photographing of bacterial colonies in Petri dishes or in tubes to demonstrate the rate of growth or their rough or smooth character on either opaque or translucent media.

With a medium that is opaque or very dark in color, there is no choice in the method of lighting. It must be by reflection, the stage being horizontal and the camera perpendicular with the lens down-

* Read at the 1934 Convention of the Biological Photographic Association.

ward. The usual photomicrographic light unit may be used with the cone of light directed diagonally downward (fig. 1). The shadows may be lightened by reflection or by a weaker supplementary light, and the height, angle, and distance may be varied, all of which has some effect on the result. As low magnifying lenses, 100 mm. to 20 mm., are used and as the illumination is by reflection, the iris diaphragm of the lens may be used to improve the depth of focus. Similar results may be obtained on a horizontal camera with the dish in a perpendicular position but then the illumination is more difficult to control.

Cultures growing on slants in tubes may be treated very much as Petri dishes on a horizontal stage. Disturbing highlights on the surface of the tubes may be minimized or obliterated by having the ends of the tubes, not the sides, toward the source of light and by raising or lowering the latter.

Cultures on opaque media in Petri dishes are sometimes studied in the laboratory with a microscope having a tilting and revolving stage. The preparation is manipulated on this stage so that the blood agar medium appears like a mirror on which the colonies are shown in relief with dark outlines and heavy shadows. The differences between rough and smooth are thus very apparent. All these conditions should be accurately reproduced by the photographer so that what appears to the eye of the observer is projected by the lens on the photographic plate. In this set-up an incandescent frosted bulb in a matte reflector is placed close to the microscope and pointed directly downward. Sometimes the light is diffused still further by placing a ground glass between the light and the preparation. This procedure requires an upright camera. To facilitate the accurate repetition of such work, exact records describing the technique should be made. (See Vol. II, No. 3, p. 166, Jour. Bio. Photo. Assoc.)

These methods of photography may be designated as: horizontal stage with reflected light and tilted stage with reflected light. The pictures resulting from these two methods are so different that the photographic technique should be recorded in the description of plates so that the reader may have a clear understanding of the meaning of the illustrations.

Colonies grown on translucent media offer the author and photog-

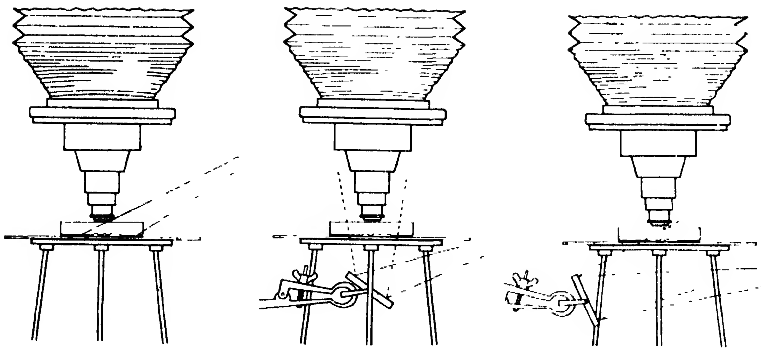


FIG. 1 FIG. 2 FIG. 3

FIG. 1. Reflected light falling directly on dish from light unit at right and above

FIG. 2. Transmitted light projected perpendicularly upward by mirrors from light unit at right

FIG. 3. Semitransmitted light projected diagonally upward by mirror through preparation but not into the lens

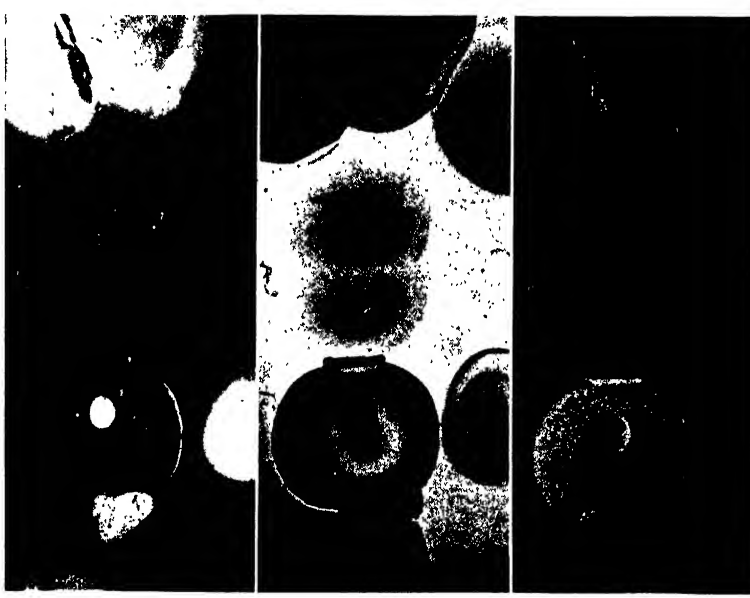


FIG. 4 FIG. 5 FIG. 6

FIGS. 4–6. Part of a dish of mixed unidentified cultures photographed in rapid succession changing only the direction of the light

Figures 1 to 3 show the corresponding methods of lighting. Wratten and Wainwright, M plates were used.

rapher a wider choice of methods. In addition to the two already described, there are at least two other means of illumination available, both employing a horizontal stage, namely, with transmitted light and with oblique transmitted or semitransmitted light. The first is the usual microscopic illumination with the light emanating from a horizontally placed unit, having a condenser, reflected perpendicularly upward by the mirror through the preparation. In the second the illumination, while transmitted, is oblique, passing upward at an angle. The reflecting mirror having been moved backward causes the light to pass through the preparation but keeps it from entering the lens. Figures 1 to 3 show the set-up for reflected, transmitted, and semitransmitted light. The last method should be used with great care, as will be seen from the illustration.

The dish of mixed, unidentified cultures photographed by the three methods of illumination resulted in prints revealing striking differences in the representation of the different colonies, their grouping and background, and other characteristics. The pictures show that certain groups of colonies very similar in one picture are split into several groups in others; that some are pale in one and dark in others, and that the granular character shown in one is entirely absent in the other two (figs. 4, 5, and 6). It is important to consider that the exposures were made in rapid succession on identical plates without filters. Only one factor was changed, namely, the direction of the light which indicates a difference in the length of exposure. Looking at the results, is it not clear that a careful description of the technique is a part of the picture? If, in addition, various filters and plates had been used, a still greater number of strikingly different effects might have been produced, further emphasizing the importance of accurate statement of photographic technique.

Frequently photographs of identical material made on widely separated dates are combined in the same article, even on the same page. Unless accurate records are kept of the first photograph of a series, and the same procedure is precisely followed in all subsequent work in the series, the resulting picture may have no value whatever. For if the method used is not stated in the description of plates, the reader is confused and in repeating the experiment or carrying on a similar investigation someone else may not obtain identical pictures. The

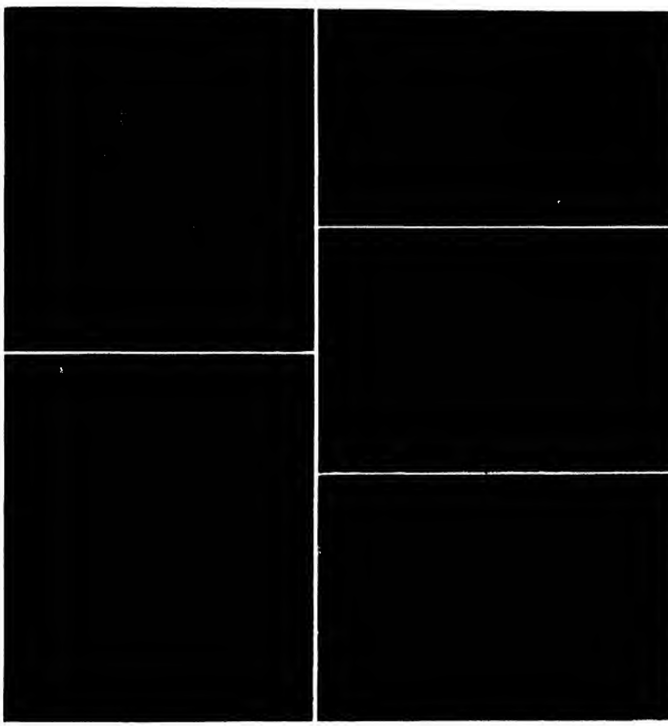
colonies may be identical in the experiment but the photographs quite different. It is conceded that good pictures are an important part of scientific communications and it is therefore urged that in most cases it will add greatly to their value if a clear description of technique is published.

Ref.

Fig. 7

Fig. 8

Semi-
Tran.



Ref.

Fig. 9

Tran.

Fig. 10

Semi-
Tran.

Fig. 11

Rough Pn. Type II. Smooth

Figs. 7–11. To show similarity of rough and smooth colonies when photographed with semitransmitted light

Figures 8 and 11, and that they look different in reflected light Figures 7 and 9

No one procedure is here advocated. Sooner or later all may be used. However, an examination of figures 8 and 11 will show that with semitransmitted light certain smooth colonies are made to appear similar to rough colonies of the same derivation which indicates, of course, that a different method should be used (see figs. 7 and 9). It goes without saying that in every study of colonies the different

variations of the same material should be photographed by identical methods.

It should be remembered that the greatest factor in photography is light and the direction it takes in relation to the object photographed. It is urged that many descriptions of plates are incomplete without the data which another photographer needs in order to obtain similar results from identical material. It is therefore suggested that in the interest of uniformity the members of this Association adopt the terms: horizontal stage, reflected light; tilted stage, reflected light; horizontal stage, transmitted or semitransmitted light.

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DETOSYLATION OF 4- AND 5-TOSYL[1] MONOACETONE *l*-METHYLRHAMNOSIDES

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Since Ferns and Lapworth[2] have pointed out the fact that the group $R-SO_2-O-$ functions as a halide atom, it is evident that the replacement of a tosyl group on an asymmetric carbon atom may be accompanied by a Walden inversion. Indeed in the sugar series such occurrences were reported first by Mathers and Robertson[3] and later by Oldham and Rutherford[4] and by Ohle and Just.[5] In all the described cases, however, opportunity existed for an ethylene oxidic ring formation as an intermediate step in the rearrangement. The question naturally arose as to whether a Walden inversion occurs in cases not permitting the oxidic ring formation. Work in this direction was initiated in this Laboratory several years ago and some of the negative results have been reported in a preliminary note.[6]

On the other hand, Muskat[7] thought he had obtained evidence in favor of the occurrence of Walden inversion on alkaline hydrolysis of 5-tosyl monoacetone *l*-methylrhamnoside. Depending upon conditions of hydrolysis, Muskat obtained either an unsaturated derivative distilling at 80° (3 mm.) or a saturated substance distilling at 100° (1 mm.). The latter product on hydrolysis was supposed to form the simple sugar, *d*-gulomethylose. The properties of the substance, its strong reducing power in the cold, mutarotation of the amorphous

(1) Term introduced by Hess and Pfleger to designate *p*-toluene sulfonyl radical [K. Hess and R. Pfleger, *Ann.*, **507**, 48 (1933)].

(2) J. Ferns and A. Lapworth, *J. Chem. Soc.*, **101**, 273 (1912).

(3) D. S. Mathers and G. J. Robertson, *ibid.*, 696, 1076 (1933).

(4) J. W. H. Oldham and J. K. Rutherford, THIS JOURNAL, **54**, 366 (1932).

(5) H. Ohle and F. Just, *Ber.*, **68**, 601 (1935).

(6) P. A. Levene and J. Compton, THIS JOURNAL, **57**, 777 (1935).

(7) I. E. Muskat, *ibid.*, **56**, 2653 (1934).

product and the sign of the equilibrium rotation did not agree with the found properties of *d*-gulomethylose.[8]

However, the possibility was not excluded that the higher boiling material obtained by Muskat was a mixture of several substances containing gulomethylose among the other products. The problem, therefore, required a rigorous reinvestigation.

The Nature of the Higher Boiling Material.—As a result of such an investigation, the conclusion was reached that the higher boiling material obtained by Muskat was slightly impure monoacetone *l*-methylrhamnopyranoside.

1. The tosyl derivative of this material obtained by Muskat with a melting point of 60°, $[\alpha]_D + 22°$ (methanol), and the same material obtained by us, on recrystallization gave a product melting at 61–62°, $[\alpha]^{24}_D +22°$ (methanol), and the mixed melting point with 4-tosyl monoacetone *l*-methylrhamnopyranoside showed no depression.

2. The same tosyl derivative on reductive detosylation by the method of Freudenberg and Brauns[9] gave a substance with the properties of monoacetone *l*-methylrhamnopyranoside.

3. On hydrolysis with dilute acid the higher boiling fraction gave a sugar whose *p*-bromophenylhydrazone had the properties of the corresponding hydrazone of rhamnose.

The Origin of the Higher Boiling Material.—The higher boiling material was not a product of a ring shift in course of the reaction but had its origin in the contamination of the monoacetone *l*-methylrhamnofuranoside with the corresponding derivative of the pyranoside. The evidence in favor of this conclusion is as follows.

1. The tosyl derivative obtained from the monoacetone *l*-methylrhamnoside prepared in the conventional way and having a melting point of 79–80° could be separated into several fractions as shown in Table I.

2. It was shown by analysis of the various hydrolytic fractions that only the substance melting at 82–83° was the pure furanoside, and that the remaining fractions were mixtures of the furanoside with pyranoside. More precisely, the results were as follows.

(*a*) The fraction melting at 82–83° on alkaline hydrolysis yielded

(8) P. A. Levene and J. Compton, *J. Biol. Chem.*, **111**, 335 (1935).

(9) K. Freudenberg and F. Brauns, *Ber.*, **55**, 3238 (1922).

practically exclusively the unsaturated product (most probably of a mannal nature) together with a minimal residue of the unchanged monoacetone *l*-methylrhamnofuranoside.

The same product on reductive hydrolysis yielded exclusively monoacetone *l*-methylrhamnofuranoside.

(*b*) The other fractions gave smaller yields of low boiling material and increasing quantities of the higher boiling fractions. From the latter fraction a tosyl derivative could be obtained melting at 61°, showing no depression of melting point when mixed with 4-tosyl monoacetone *l*-methylrhamnopyranoside. On reductive hydrolysis this tosyl derivative yielded a product identical with monoacetone *l*-methylrhamnopyranoside.

(*c*) Pure 4-tosyl monoacetone *l*-methylrhamnopyranoside, m.p. 61–62°, on alkaline hydrolysis, did not yield the unsaturated derivative, but gave exclusively the higher-boiling material which, on retosylation, yielded a tosyl derivative with the properties of the original material.

Thus, it is evident that monotosyl derivatives of rhamnose, in which the opportunity for oxide formation is excluded, behave similarly to the tosyl derivatives of other sugars, namely, they undergo alkaline detosylation without Walden inversion.[10]

It may be added that the two tosyl derivatives with melting point of 82–83° and of 61–62° were shown definitely to have the furanoside and the pyranoside ring structures, respectively.

From the experience gained during this work it is found that absolutely pure monoacetone *l*-methylrhamnofuranoside is obtained most advantageously by reductive detosylation of the tosyl derivative melting at 82–83°.

EXPERIMENTAL

A. Preparations

Monoacetone Methylrhamnofuranoside.—Two methods were employed in the preparation of this material, each resulting in the simultaneous formation of monoacetone methylrhamnopyranoside as an impurity.

(a) Fifty grams of anhydrous rhamnose[11] was suspended in 1000 cc. of acetone

(10) J. W. H. Oldham and G. J. Robertson, *J. Chem. Soc.*, **685** (1935).

(11) P. A. Levene and I. E. Muskat, *J. Biol. Chem.*, **105**, 761 (1934).

containing 5% of absolute methyl alcohol. Anhydrous copper sulfate (100 g.) was then added and sufficient concentrated sulfuric acid to make the solution 0.2%. The mixture was shaken at room temperature for twenty hours, after which it was worked up as previously described; total yield of monoacetone methylrhamnosides, 45.1 g., having the same specific rotations and refractive indices as previously recorded.[11]

(b) Fifty grams of monoacetone rhamnose[12] was dissolved in 1000 cc. of absolute methyl alcohol containing 1% of anhydrous hydrogen chloride. The mixture was allowed to stand at room temperature overnight, after which it was allowed to reflux on the steam-bath for thirty minutes. The solution was made neutral with excess silver carbonate, filtered and concentrated under diminished pressure to a thin sirup which distilled completely under diminished pressure; yield, 40 g.; b. p. 99–100° (0.8 mm.); $n^{25}D$ 1.4485; $[\alpha]^{25}D$ −63.0° (*c*, 3.379, methyl alcohol); $[\alpha]^{25}D$ −45.5° (*c*, 2.504, water).

Anal. Calcd. for $C_{10}H_{18}O_5$: OCH_3, 14.22. Found: OCH_3, 14.22.

TABLE I

Fractionation of Tosyl Derivatives of Monoacetone Methylrhamnoside

Fraction	M.p., °C.	$[\alpha]^{25}D$ in methanol
I	82–83 (max.)	−13.6° (*c*, 3.168)
II	74–75	− 5 6° (*c*, 3.046)
III	68–69	− 1.7° (*c*, 2.964)
IV	64–65	+ 5.6° (*c*, 3.030)
V	57–58	+12.1° (*c*, 2.708)

There remained after distillation of the monoacetone methylrhamnosides 8 g. of unchanged monoacetone rhamnose, b. p. 130–136° (0.8 mm.), and a small amount (0.5 g.) of methylrhamnopyranoside, b. p. 148–154° (0.5 mm.).

5-Tosyl[1] Monoacetone Methylrhamnofuranoside.—This compound was prepared according to the procedure of Levene and Muskat,[11] with slight modifications.

Monoacetone methylrhamnoside (20 g.) prepared as described in method (a) or (b) above, was dissolved in 30 cc. of dry pyridine and 32 g. of tosyl chloride added. Shaking was maintained until solution was complete, after which the mixture was allowed to stand overnight at room temperature. The mixture was then thoroughly cooled and 2 cc. of water added. After standing for thirty minutes the partially crystallized mass was poured with vigorous stirring into 250 cc. of ice-cold saturated sodium bicarbonate solution. The sirupy product first separating soon solidified and was removed by filtration, washed thoroughly with water and dried; yield 22 g. The product recrystallized from methyl alcohol

(12) K. Freudenberg, *Ber.*, 59, 836 (1926).

had a melting point of 80–81°. After a second and third recrystallization, a constant melting point of 82–83° was obtained; $[\alpha]^{26}D$ −13.6° (*c*, 3.168, methyl alcohol). Continued recrystallization of the mixed crystals obtained from the mother liquors gave fractions having the properties shown in Table I.

Monoacetone Methylrhamnopyranoside.—Acetonation of methylrhamnopyranoside according to the procedure of Levene and Muskat[13] led to the formation of pure monoacetone methylrhamnopyranoside, b.p. 104–105° (0.8 mm.); $n^{24}D$ 1.4545; $[\alpha]^{24}D$ −11.9° (*c*, 3.366, methanol).

4-Tosyl Monoacetone Methylrhamnopyranoside.—Twenty grams of monoacetone methylrhamnopyranoside was tosylated and the product isolated in the manner described for 5-tosyl monoacetone methylrhamnofuranoside; yield 25.5 g.; melting point after one recrystallization from methyl alcohol, 61–62°. Further recrystallizations from this solvent changed neither the melting point nor specific rotation; $[\alpha]^{24}D$ + 21.94° (*c*, 3.030 methanol).

Anal. Calcd. for $C_{17}H_{24}O_7S$: C, 54.80; H, 6.45; S, 8.60. Found: C, 54.98; H, 6.46; S, 8.69.

B. Alkaline Hydrolysis

1. Pure 5-Tosyl Monoacetone Methylrhamnofuranoside.—(Fraction I, Table I.) Forty grams of pure 5-tosyl monoacetone methylrhamnofuranoside (m. p. 82–83°; $[\alpha]^{25}D$ −13.5°) was dissolved in 1500 cc. of methyl alcohol and 120 g. (20 moles) of potassium hydroxide dissolved in 1300 cc. of water was added to the boiling solution. The reaction was 95% complete after refluxing on the steam-bath for sixty hours at 78° after which the solution was cooled and the excess of alkali exactly neutralized by means of 25% hydrochloric acid. Sufficient calcium chloride was then added to saturate the solution, after which it was exhaustively extracted with chloroform. The chloroform extract was allowed to stand over calcium chloride for twenty-four hours, filtered, and concentrated under diminished pressure. The resulting sirup distilled in two fractions: *first*, b. p. 56–60° (0.3 mm.), yield 16.0 g. $n^{24}D$ 1.4491; *second*, b. p. 82–85° (0.3 mm.), yield 0.8 g., $n^{4}D$ 1.4501.

Anal. First fraction. Calcd. for $C_{10}H_{16}O_4$: C, 59.97; H, 8.0; OCH_3, 15.49. Found: C, 59.64; H, 8.21; OCH_3, 15.21. *Second fraction.* Calcd. for $C_{10}H_{18}O_5$: C, 55.02; H, 8.30; OCH_3, 14.22. Found: C, 54.85; H, 8.38; OCH_3, 14.42.

A portion of the second fraction (0.6 g.) (b. p. 82–85° at 0.3 mm.), was dissolved in 1 cc. of dry pyridine and 0.8 g. of tosyl chloride added. After standing overnight at room temperature the solution was worked up as described for the tosylation of monoacetone methylrhamnofuranoside (yield 0.65 g.; m.p. 77–78°). After one recrystallization from methyl alcohol a constant melting point of 82–83° was obtained which was unchanged on admixture with an authentic specimen of 5-tosyl monoacetone methylrhamnofuranoside; specific rotation of this material $[\alpha]^{25}D$ −13.05° (*c*, 3.064, methanol).

(13) P. A. Levene and I. E. Muskat, *J. Biol. Chem.*, **105**, 431 (1934).

The various conditions employed in attempts to obtain from pure 5-tosyl monoacetone methylrhamnofuranoside the product described by Muskat[7] are summarized in Table II.

2. Mixed 4- and 5-Tosyl Monoacetone Methylrhamnosides, m. p. 68°.—(Fraction III, Table I.) Four grams of mixed crystals of 4- and 5-tosyl monoacetone methylrhamnosides (m. p. 68°) was dissolved in 200 cc. of methyl alcohol and 12 g. of potassium hydroxide dissolved in 140 cc. of water added on the steam-bath. Refluxing was maintained for seventy-five hours, when the reaction was complete. After cooling, the hydrolysis products were isolated in the manner described above (1). Two fractions were obtained: first, b. p. 58–60° (0.3 mm.), yield 0.6 g.; second, b. p. 82–85° (0.3 mm.), yield 1.1 g.

TABLE II

Effect of Alkaline Hydrolysis Mixtures on Pure 5-Tosyl Monoacetone Methylrhamnofuranoside (m. p. 82–83°)

Expt.	Wt., g.	KOH, moles	Methanol, cc.	Water, cc.	Temp., °C.	Time, hours	% Yield of unsatd. compd. of b. p. 60° (0.3 mm.)
I	4	20	200	40	74	50	95
II	2	20	100	0	100	30	90
III	4	20	160	180	80	48	95
IV	2	2	200	20	125	18	90
V	2	2	80	60	100	18	90
VI	2	20	100	40	37	2 mo.	No reaction
VII	2	20 ($NaOCH_3$)	100	30	70	50	98

Retosylation of the high boiling sirup (0.7 g.) with tosyl chloride (1.2 g.) in the presence of pyridine (1.0 cc.) gave a tosyl compound (0.8 g.) melting at 59–60°. After one recrystallization from methyl alcohol a constant melting point of 60–61° was obtained; $[\alpha]^{25}_{D}$ + 20.2° (*c*, 2.868, methanol). A mixed melting point of this material with pure 4-tosyl monoacetone methylrhamnofuranoside (m. p. 61–62°) showed no depression.

3. Impure 5-Tosyl Monoacetone Methylrhamnofuranoside, m. p. 79–80°.—Inasmuch as the 5-tosyl monoacetone methylrhamnofuranoside (m. p. 80°) previously reported[10] had a lower melting point than that employed in experiment 1, a sample of this compound (unfractionated) with m. p. 79–80° was used: 40 g. of 5-tosyl monoacetone methylrhamnoside (m. p. 79–80°) was dissolved in 1500 cc. of methyl alcohol and 120 g. of potassium hydroxide dissolved in 1300 cc. of water added on the steam-bath. The temperature (80°) was maintained for fifty hours when the reaction was about 95% complete. After cooling, the solution was worked up as described above; yield in three fractions: first, 17.1 g., second, 1.5 g., and third, 3.0 g. with b. p. 58–60° (0.3 mm.), 80–82° (0.3 mm.), and 83–85° (0.3 mm.), respectively.

The first fraction consisted of the unsaturated compound, whereas the second

consisted of a mixture of monoacetone methylfuranoside and monoacetone methylpyranoside and the third was pure pyranoside, as shown on retosylation.

The second fraction (b. p. 80–82° at 0.3 mm.) was dissolved in 2.5 cc. of dry pyridine and 2.2 g. of tosyl chloride added. The mixture was allowed to stand overnight, after which it was worked up as described above; yield 1.4 g. Recrystallization from methyl alcohol gave a product (m. p. 58°) which after the fourth crystallization had risen to 67–68° $[\alpha]^{27}_D$ 0.00° (*c*, 2.976 methanol). From the mother liquors there was obtained a small amount of mixed 4- and 5-tosyl compounds, m. p. 55–58°.

The third fraction (b. p. 83–85° at 0.3 mm.) was divided into two parts. (a) A portion of this material (0.5 g.) was retosylated with tosyl chloride (0.8 g.) in the presence of dry pyridine (1.2 cc.) by allowing to stand overnight at room temperature; yield 0.6 g. After one recrystallization a substance of constant melting point of 60–61° was obtained which when mixed with pure 4-tosyl monoacetone methylrhamnofuranoside showed no depression; specific rotation of this material, $[\alpha]^{24}_D$ + 22.1° (*c*, 3.032 methanol).

Anal. Calcd. for $C_{17}H_{24}O_7S$: C, 54.80; H, 6.45; S, 8.60. Found: C, 54.60; H, 6.30; S, 8.72.

(b) A second portion of this material (third fraction) (1.0 g.) was dissolved in 40 cc. of 1% sulfuric acid solution and allowed to reflux slowly for ninety minutes. The solution was rendered exactly neutral with barium hydroxide, treated with charcoal, and filtered. Upon concentrating under diminished pressure at 40° there was obtained 0.8 g. of sirupy material which on heating reduced Fehling's solution.

Four-tenths gram of this sirup was converted to the *p*-bromophenylhydrazone using one mole of *p*-bromophenylhydrazine in 5 cc. of absolute ethyl alcohol. There was thus obtained 0.35 g. of material, m. p. 168°, unchanged by further recrystallization from ethyl alcohol. A mixed melting point of this material with an authentic specimen of rhamnose *p*-bromophenylhydrazone (m. p. 167–168°) showed no depression. The mutarotation of the above *p*-bromophenylhydrazone in ethyl alcohol was in the same direction as that of rhamnose *p*-bromophenylhydrazone.

$$[\alpha]^{25}_D\ -11.29^\circ \xrightarrow{\text{24 hrs.}} +5.8^\circ \xrightarrow{\text{2 weeks}} -21.29^\circ \text{ the latter,}$$

$$[\alpha]^{23}_D\ -12.5^\circ \xrightarrow{\text{30 hrs.}} +10.2^\circ \xrightarrow{\text{2 weeks}} -23.1^\circ$$

No other *p*-bromophenylhydrazone was found in the reaction product, thus showing that rhamnose was the only sugar present in the third fraction.

Anal. Calcd. for $C_{12}H_{17}O_4N_2Br$: C, 43.24; H, 5.14; N, 8.40. Found: C, 43.35; H, 5.02; N 8.61.

4. *Pure 4-Tosyl Monoacetone Methylrhamnopyranoside.*—Four grams of 4-tosyl monoacetone methylrhamnopyranoside (m. p. 61–62°) was dissolved in 150 cc. of methyl alcohol and 130 cc. of water containing 12 g. (20 moles) of potassium hydroxide added to the solution on the steam-bath. After refluxing for sixty

hours, hydrolysis was complete and the excess alkali was neutralized with carbon dioxide. The solution was evaporated to dryness at 40° under diminished pressure and the resulting solid thoroughly extracted with ether. The ether extract was dried over calcium chloride, filtered, and concentrated under diminished pressure to a thick sirup which distilled completely, b. p. 84–85° (0.3 mm.); yield 2.0 g.; $[\alpha]^{25}_D$ −20.5° (*c*, 3.230, methanol); n^{24}_D 1.4544.

Anal. Calcd. for $C_{10}H_{18}O_5$: C, 55.02; H, 8.30; OCH_3, 14.22. Found: C, 55.12; H, 8.40; OCH_3, 14.35.

Retosylation of monoacetone methylrhamnopyranoside thus obtained (0.86 g.) with tosyl chloride (1.5 g.) in the presence of dry pyridine (1.2 cc.) followed by isolation and crystallization as previously described yielded 0.90 g. of material, m. p. 61–62°. A mixed melting point of this material with 5-tosyl monoacetone methylrhamnopyranoside showed no depression. Specific rotation $[\alpha]^{25}_D$ +21.8° (*c*, 3.028, methanol).

C. Reductive Alkaline Hydrolysis

1. Pure 5-Tosyl Monoacetone Methylrhamnofuranoside.—(Fraction I, Table I.) *Two grams of 5-tosyl monoacetone methylrhamnofuranoside (m.p. 82–83°) was dissolved in 100 cc. of 80% methyl alcohol and 25 g. of 4%* sodium amalgam added under good stirring. After fourteen hours at room temperature the reaction was complete. The excess alkali was neutralized with a stream of carbon dioxide and the solution evaporated to dryness at 40° under diminished pressure. The resulting solid was thoroughly extracted with hot ether and the extract dried over calcium chloride, filtered, and concentrated under diminished pressure; yield, 1.1 g. with b. p. 78–80° (0.2 mm.); $[\alpha]^{21}_D$ −75.45° (*c*, 2.770, methanol); n^{24}_D 1.4466.

Anal. Calcd. for $C_{10}H_{18}O_5$: C, 55.02; H, 8.30; OCH_3, 14.22. Found: C, 54.99; H, 8.35; OCH_3, 14.21.

Retosylation of 0.5 g. of this pure material with 0.8 g. of tosyl chloride dissolved in 1 cc. of dry pyridine in the usual manner, gave a yield of 0.6 g. of 5-tosyl monoacetone methylrhamnofuranoside (m. p. 82–83°). The melting point was unchanged when mixed with an authentic specimen of 5-tosyl monoacetone methylrhamnofuranoside.

2. Mixed 4- and 5-Tosyl Monoacetone Methylrhamnosides, m. p. 57–58°.—Fraction V, Table I. The mixed crystals of 4- and 5-tosyl monoacetone methylrhamnosides (2.5 g.) dissolved in 250 cc. of 80% methyl alcohol were detosylated by use of 36 g. of 4% Na-Hg amalgam during fourteen hours. The product isolated as described above had a boiling point of 82–85° (0.3 mm.); yield 1.1 g.; $[\alpha]^{25}_D$ −38.18° (*c*, 3.156, methanol); n^{24}_D 1.4520.

3. Pure 4-Tosyl Monoacetone Methylrhamnopyranoside.—(a) Ten grams of 4-tosyl monoacetone methylrhamnopyranoside was dissolved in 500 cc. of 80% methyl alcohol and 125 g. of 4% Na-Hg amalgam added. Good stirring was maintained for fourteen hours, after which the reaction was complete. The solution was worked up and the product isolated as previously described; yield 5.4 g.; b. p. 84–85 (0.3 mm.); $[\alpha]^{25}_D$ −17.76° (*c*, 3.294, methanol); n^{24}_D 1.4541.

Anal. Calcd. for $C_{10}H_{18}O_5$: C, 55.02; H, 8.30; OCH_3, 14.22. Found: C, 54.94; H, 8.41; OCH_3, 14.00.

TABLE III

Hydrolysis of Monoacetone Methylrhamnosides with 0.1 N HCl at 100°

Monoacetone methylrhamnoside obtained by	% Solution	$[\alpha]^{26}_D$ Initial	$[\alpha]^{26}_D$ After 30 min.
(1) Reductive hydrolysis of tosyl derivative of m. p. 82–83°[a]	3.346	−58.0°	+ 5.0°
(2) Reductive hydrolysis of tosyl derivative of m. p. 61–62°[b]	4.280	− 6.0°	−35.8°
(3) Simultaneous methylation and acetonation of rhamnose, or "methylation" of monoacetone rhamnose[b]	2.830	−45.5°	− 8.4°
(4) Acetonation of methylrhamnopyranoside[b]	3.236	− 4.8°	−35.1°

[a] Final value of specific rotation calculated as free rhamnose.

[b] Final values of specific rotations calculated as methylrhamnosides.

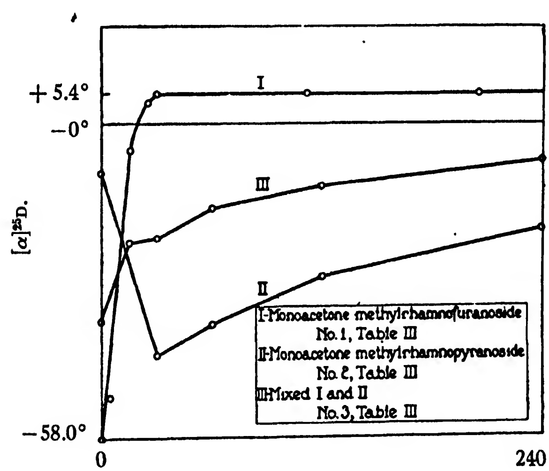

FIG. 1.—Rate of hydrolysis of monoacetone methylrhamnosides with 0.03 *N* HCl at 100°.

Retosylation of 0.5 g. of this material with tosyl chloride (0.8 g.) in presence of dry pyridine (1 cc.) yielded in the usual manner, 0.6 g. of material of m. p. 61–62°; $[\alpha]^{26}_D$ + 21.88° (*c*, 3.014, methanol). A mixed melting point with original 4-tosyl monoacetone methylrhamnopyranoside showed no depression.

(b) The alkaline hydrolysis of mixed crystals of 4- and 5-tosyl methylrhamnosides (m. p. 68 or 80°) leads to the production of an unsaturated low boiling material and monoacetone methylrhamnopyranoside which in turn, upon tosylation, yields 4-tosyl monoacetone methylrhamnopyranoside (m. p. 60–61°). To prove further that this material was identical with 4-tosyl monoacetone methylrhamnopyranoside, a sample thus obtained was detosylated and the properties of the monoacetone methylrhamnopyranoside obtained were compared with those of the known derivative of this structure.

4-Tosyl monoacetone methylrhamnopyranoside (0.8 g.) with m. p. 60–61° *was dissolved in 80 cc. of 80% methyl alcohol and 12 g. of 4%* Na-Hg amalgam *added. The time of reaction and method of isolation was carried out exactly as described previously; yield 0.4 g.; b. p. 82–85° (0.3 mm.); $[\alpha]^{25}_D$ −21.30° (c, 3.380, methanol); n^{24}_D 1.4539.*

Anal. Calcd. for $C_{10}H_{18}O_5$: C, 55.02; H, 8.30; OCH_3, 14.22. Found: C, 55.03; H, 8.19; OCH_3, 14.26.

D. Proof of Structure of 4- and 5-Tosyl Monoacetone Methylrhamnosides

Inasmuch as the methylglycofuranosides are hydrolyzed at a much greater rate than the glycopyranosides,[14] the ring structure of such compounds may be determined easily. Accordingly the hydrolysis rates of the monoacetone methylrhamnosides (purified and crude) were determined in the following manner. Known weights of the monoacetone methylrhamnosides were dissolved in hydrochloric acid (0.1 or 0.03 *N*) and diluted to exactly 5 cc. with hydrochloric acid (0.1 or 0.3 *N*). The specific rotation was observed immediately and the solution then heated in a sealed tube at 100° for the time specified. The tubes were now cooled and the rotation of the solution again noted. The results obtained in this manner are given in Table III and in Fig. 1.

We wish to express our thanks and gratitude to Messrs. Geigy and Company for their generous gift of the *l*-rhamnose used in these experiments.

SUMMARY

1. The usual methods of preparing monoacetone *l*-methylrhamnofuranoside are attended by the simultaneous formation of monoacetone *l*-methylrhamnopyranoside, at times to the extent of 30–40%.

2. Alkaline hydrolysis of pure 5-tosyl monoacetone *l*-methylrhamnofuranoside leads to the production of an unsaturated compound in 90–95% yield together with monoacetone *l*-methylrhamnofuranoside.

(14) P. A. Levene and G. M. Meyer, *J. Biol. Chem.*, **78**, 363 (1928); H. G. Bott, E. L. Hirst and J. A. B. Smith, *J. Chem. Soc.*, 658 (1930).

3. Alkaline hydrolysis of 4-tosyl monoacetone *l*-methylrhamnopyranoside yields quantitatively monoacetone methylrhamnopyranoside.

4. Tosyl monoacetone *l*-methylrhamnoside with m.p. 80° is a mixture containing, in addition to the furanoside, a small proportion of the pyranoside. Alkaline hydrolysis of this product leads to formation of the unsaturated derivative together with a small proportion of monoacetone *l*-methylrhamnopyranoside.

5. Alkaline hydrolysis of the 4- and 5-tosyl monoacetone *l*-methylrhamnosides is accomplished without Walden inversion.

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THE STRUCTURE OF *d*-XYLOMETHYLOSE

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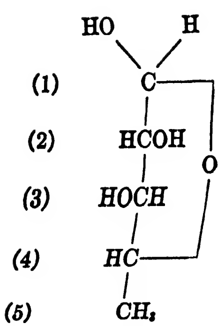
In previously assigning to *d*-xylomethylose[1] the structure given in Formula I, the following assumptions have been made: first, that the structure of monoacetone xylose is represented by Formula III; second, that in the unimolecular tosylation of monoacetone xylose the primary hydroxyl is preferentially esterified, and third, that the replacement of the tosyl group by iodine and subsequent reduction of the latter to a desoxy derivative cause no fundamental structural changes. The work of Haworth and Porter[2] establishes conclusively that Formula III represents the structure of monoacetone xylose. The second and the third assumptions have been based on the experience previously noted in the case of certain hexoses. In the present communication, experimental evidence is presented which demonstrates the validity of the above assumptions and which therefore definitely establishes the structure (I) assigned by us to *d*-xylomethylose.

The problem of determining the structure of "*d*-xylomethylose" resolves itself into the correct allocation of the desoxy group. Since the acetone rest in monoacetone xylomethylose unquestionably occupies positions (1) and (2), the only remaining positions which the desoxy group may occupy are (3), (4), and (5). Position (4) must be considered, since the possibility of a ring shift is not excluded *a priori*. In assigning the desoxy group to position (5), the following lines of evidence are presented. First, nitric acid oxidation of completely methylated "xylomethylose" (Formulas IV and V) yields exclusively dimethyl *d*-tartaric acid (Formula VI). This result can only be explained when the desoxy

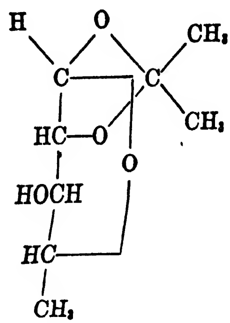
[1] Levene, P. A., and Compton, J., *J. Biol. Chem.*, **111**, 325 (1935).

[2] Haworth, W. N., and Porter, C. R., *J. Chem. Soc.*, 611 (1928).



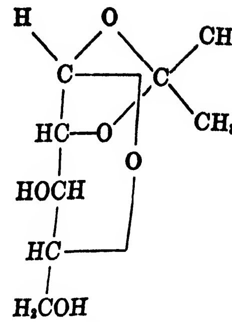
I

d-Xylomethylose



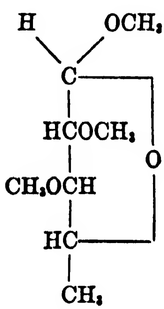
II

Monoacetone *d*-xylomethylose



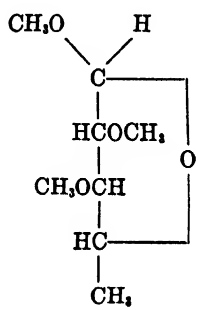
III

Monoacetone *d*-xylose



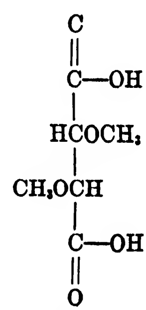
IV

2,3-Dimethyl α-methyl-*d*-xylomethyloside



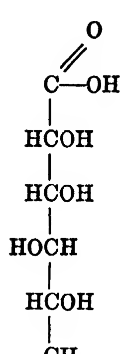
V

2,3-Dimethyl β-methyl-*d*-xylomethyloside



VI

Dimethyl *d*-tartaric acid



VII

d-Gulomethylonic acid

group is in position (5), since a desoxy group in either position (3) or (4) would lead to the formation of a dimethoxy glutaric acid.[3,4,5] Second, the oxidation of "*d*-xylomethylose" with silver oxide yields only silver acetate.[4,6] Third, the polarimetric observation of the rate of lactone formation of *d*-gulomethylonic acid[7] (Formula VII) indicates the formation of both γ- and δ-lactones.

Completely methylated *d*-xylomethylose was obtained by the stepwise methylation of *d*-xylomethylose in a manner involving the least possible structural change. Crystalline monoacetone *d*-xylomethylose was methylated with Purdie's reagent to yield 3-methyl monoacetone *d*-xylomethylose, a colorless mobile liquid, b.p. 58–60° at 0.3 mm., $[\alpha]_D^{25} = -49.4°$. Acid hydrolysis of this compound yielded 3-methyl xylomethylose, isolated as a thick sirup, b.p. 100–102° at 0.8 mm., $[\alpha]_D^{25} = +8.1°$. Treatment of 3-methyl xylomethylose with dry methyl alcohol-hydrogen chloride (1 per cent) led to the simultaneous formation of 3-methyl α-methylxylomethyloside, b.p. 58–62° at 0.3 mm., $[\alpha]_D^{25} = +124.5°$, and crystalline 3-methyl β-methylxylomethyloside, m.p. 48–50°, $[\alpha]_D^{25} = -127.9°$. Further methylation of the α and β isomers with Purdie's reagent led to the formation of crystalline 2,3-dimethyl α-methylxylomethyloside (Formula IV), m.p. 34–35°, $[\alpha]_D^{26} = +154.0°$, and 2,3-dimethyl β-methylxylomethyloside (Formula V), b.p. 38–40° at 0.5 mm., $[\alpha]_D^{26} = -102.4°$. Oxidation of the completely methylated α,β pair with concentrated nitric acid yielded exclusively in each case dimethyl *d*-tartaric acid (Formula VI), identified in the case of the α isomer oxidation as the free acid (m.p. 151–152°) and in the case of the β isomer as the dimethylamide derivative (m.p. 205–206°).

EXPERIMENTAL

3-Methyl Monoacetone d-Xylomethylose—Monoacetone *d*-xylomethylose (7.0 gm.) was dissolved in 70 cc. of methyl iodide and 40 gm. of silver oxide were added in small portions over a period of 4 hours with stirring at 50°. The mixture was then filtered and

[3] Kiliani, H., *Ber. chem. Ges.*, **38**, 4040 (1905).
[4] Micheel, F., *Ber. chem. Ges.*, **63**, 347 (1930).
[5] Elderfield, R. C., *J. Biol. Chem.*, **111**, 527 (1935).
[6] Kiliani, H., *Ber. chem. Ges.*, **32**, 2197 (1899).
[7] Levene, P. A., and Compton, J., *J. Biol. Chem.*, **111**, 335 (1935).

the silver residues thoroughly extracted with hot acetone. The thick sirup, obtained after concentrating the combined filtrates under diminished pressure, distilled completely under high vacuum. B.p. 58–60° at 0.3 mm. Yield 6.2 gm. n_D^{22} 1.4377. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{-2.86^\circ \times 100}{2 \times 2.892} = -49.4^\circ$$

The composition agreed with that of a methyl monoacetone pentomethylose.

5.422 mg. substance: 11.430 mg. CO_2 and 4.168 mg. H_2O			
5.380 " " : 6.702 " AgI			
	$C_9H_{16}O_4$.	Calculated.	C 57.40, H 8.57, OCH_3 16.48
	188.1	Found.	" 57.48, " 8.60, " 16.43

The substance is soluble in all the usual organic solvents and in water.

3-Methyl d-Xylomethylose—3-Methyl monoacetone *d*-xylomethylose (5.2 gm.) was dissolved in 100 cc. of 1 per cent sulfuric acid and heated on a boiling water bath for 60 minutes. The initial rotation of $[\alpha]_D^{25} = -48.0^\circ$ had changed at the end of this time to the constant value $[\alpha]_D^{25} = +8.8^\circ$. The sulfuric acid was then exactly neutralized with barium hydroxide and the solution treated with charcoal and filtered. The clear filtrate was concentrated under diminished pressure to a sirup which was dried by the repeated addition of absolute ethyl alcohol, followed by dry benzene. The resulting sirup was dissolved in dry ether and dried over anhydrous sodium sulfate. After filtering, the ether solution was concentrated under diminished pressure to a sirup which distilled completely under high vacuum. B.p. 100–102° at 0.8 mm. Yield 3.1 gm. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{+0.32^\circ \times 100}{1 \times 3.936} = +8.1^\circ$$

remaining constant. The composition agreed with that of a methyl pentomethylose.

4.900 mg. substance: 8.695 mg. CO_2 and 3.690 mg. H_2O			
4.900 " " : 7.740 " AgI			
	$C_6H_{12}O_4$.	Calculated.	C 48.61, H 8.17, OCH_3 20.93
	148.1	Found.	" 48.39, " 8.42, " 20.64

The substance reduced Fehling's solution strongly when heated. It is soluble in organic solvents and in water.

3-Methyl d-Xylomethylose Phenylosazone—3-Methyl xylomethylose (0.15 gm.) was dissolved in 20 cc. of water and 0.5 gm. of phenylhydrazine dissolved in 3 cc. of glacial acetic acid were added. After heating at 100° for 10 minutes the osazone began to separate and upon cooling the solution, it crystallized as yellow flocks. After filtering, the product was recrystallized from 50 per cent methyl alcohol to give the constant melting point, 128–130°. The composition agreed with that of a methyl pentomethylose phenylosazone.

4.840 mg. substance: 11.750 mg. CO_2 and 2.980 mg. H_2O
7.350 " " : 5.260 " AgI

$C_{18}H_{22}O_2N_4$.	Calculated.	C 66.22, H 6.79, OCH_3 9.50
326.18	Found.	" 66.20, " 6.88, " 9.44

3-Methyl α- and β-Methylxylomethylosides—3-Methyl *d*-xylomethylose (2.4 gm.) was dissolved in 50 cc. of absolute methyl alcohol containing 1 per cent of dry hydrogen chloride and allowed to reflux on the steam bath for 1 hour. The initial specific rotation of $[\alpha]_D^{25} = +10.2°$ had changed at the end of this time to the constant value of $[\alpha]_D^{25} = +5.1°$. The acid was now removed with excess silver carbonate and the resulting clear filtrate concentrated under diminished pressure to a clear mobile sirup which distilled in two fractions under high vacuum. The first fraction was the pure α isomer, b.p. 58–62° at 0.3 mm. Yield 0.9 gm. n_D^{25} 1.4410. The second fraction was the pure β isomer, b.p. 72–75° at 0.3 mm., which crystallized spontaneously during the distillation. M.p. 48–50°. Yield 1.0 gm. The high vapor pressure of the α isomer makes the separation from the β isomer quite easy. The specific rotation of 3-methyl α-methylxylomethyloside in water was

$$[\alpha]_D^{25} = \frac{+3.75° \times 100}{1 \times 3.012} = +124.5°$$

and that of 3-methyl β-methylxylomethyloside in water was

$$[\alpha]_D^{25} = \frac{-4.20° \times 100}{1 \times 3.284} = -127.9°$$

The composition of both substances agreed with that of an α, β pair of a methyl methylpentomethyloside.

6.112 mg. α isomer:	11.585 mg. CO_2 and 4.780 mg. H_2O		
3.615 " " " :	10.485 " AgI		
5.232 " β " :	9.950 " CO_2 and 4.070 mg. H_2O		
6.320 " " " :	18.315 " AgI		
$C_7H_{14}O_4$.	Calculated.	C 51.81, H 8.70, OCH_3 38.27	
162.11	Found.		
	α isomer.	" 51.69, " 8.75, " 38.28	
	β "	" 51.86, " 8.70, " 38.25	

Neither the α nor β isomer reduced boiling Fehling's solution but after acid hydrolysis both gave a strong test. The isomeric substances are soluble in organic solvents and in water.

2,3-Dimethyl α-Methylxylomethyloside—3-Methyl α-methylxylomethyloside (0.8 gm.) was methylated with methyl iodide (12 cc.) in the presence of silver oxide (10 gm.) in the manner previously described. Distillation of the sirupy liquid under high vacuum showed that the substance was homogeneous. B.p. 39–41° at 0.5 mm. The product crystallized completely after distillation. M.p. 34–35°. Yield 0.7 gm. The specific rotation in water was

$$[\alpha]_D^{20} = \frac{+1.83° \times 100}{1 \times 1.188} = +154.0°$$

The composition of the substance agreed with that of a dimethyl methylpentomethyloside.

4.400 mg. substance:	8.795 mg. CO_2 and 3.560 mg. H_2O	
3.690 " " :	14.720 " AgI	
$C_8H_{16}O_4$.	Calculated.	C 54.50, H 9.15, OCH_3 52.80
176.13	Found.	" 54.51, " 9.05, " 52.65

Preparation of Dimethyl d-Tartaric Acid from 2,3-Dimethyl α-Methylxylomethyloside—0.4 gm. of 2,3-dimethyl α-methylxylomethyloside (m.p. 34–35°) was dissolved in 5 cc. of concentrated nitric acid (1.42) and placed in a water bath at 50°. The temperature was gradually raised over a period of 30 minutes to 100°, after which it was maintained at 95–100° for 7 hours. At the end of this time the solution was concentrated under diminished pressure to a solid crystalline mass at 40°. The last traces of nitric acid were then removed under high vacuum by placing

the crystalline material in a vacuum desiccator over solid sodium hydroxide for 3 hours. The crude product was then dissolved in dry ether and filtered. The clear filtrate, upon concentrating under diminished pressure, crystallized completely and was filtered off and washed several times with dry ether. The melting point of the purified material was 151–152°, which was unchanged when mixed with an authentic specimen of dimethyl *d*-tartaric acid.[8] Yield 0.3 gm. The specific rotation in water was

$$[\alpha]_D^{25} = \frac{+1.43° \times 100}{1 \times 1.992} = +71.8°$$

2,3-Dimethyl β-Methylxylomethyloside—3-Methyl β-methylxylomethyloside (0.8 gm.) was methylated with methyl iodide (12 cc.) in the presence of silver oxide (10 gm.) in the usual manner. The mobile sirup thus obtained distilled completely under high vacuum, b.p. 38–40° at 0.5 mm. n_D^{25} 1.4261. Yield 0.6 gm. The specific rotation in water was

$$[\alpha]_D^{26} = \frac{-1.91° \times 100}{1 \times 1.866} = -102.4°$$

The composition agreed with that of a dimethyl methylxylomethyloside.

5.380 mg. substance:	10.735 mg. CO_2 and 4.310 mg. H_2O		
4.592 " " :	18.240 " AgI		
$C_8H_{16}O_4$.	Calculated.	C 54.50, H 9.15, OCH_3 52.80	
176.13	Found.	" 54.51, " 8.96, " 52.21	

Preparation of d-Dimethoxysuccinomethylamide from 2,3-Dimethyl β-Methylxylomethyloside—The oxidation of 2,3-dimethyl β-methylxylomethyloside (0.4 gm.) with concentrated nitric acid (5 cc.) to yield dimethyl *d*-tartaric acid was carried out as described above for the α isomer. In this case the reaction product was esterified by refluxing for 6 hours with absolute methyl alcohol (20 cc.) containing 2 per cent dry hydrogen chloride. At the end of this time the acid was neutralized with excess silver carbonate and the solution filtered. The sirup obtained upon concentrating the filtrate under diminished pressure was dissolved in dry ether,

[8] Purdie, T. P., and Irvine, J. C., *J. Chem. Soc.*, 79, 959 (1901).

dried over anhydrous sodium sulfate, filtered, the solvent evaporated, and the product distilled under high vacuum. B.p. 78–80° at 0.3 mm. Yield 0.5 gm.

The material thus obtained was dissolved in 5 cc. of absolute methyl alcohol, cooled to 0°, and saturated with dry methylamine. After standing for 1 day at 0° the solution was concentrated under diminished pressure, whereupon the amide crystallized completely and was purified by recrystallizing from ethyl acetate. M.p. 205–206°. A mixed melting point of this material with an authentic specimen of *d*-dimethoxysuccinomethylamide[9] (m.p. 205–206°) showed no depression.

Oxidation of d-Xylomethylose with Silver Oxide—*d*-Xylomethylose (1.0 gm.) was dissolved in 50 cc. of water containing a suspension

TABLE I

Rate of Lactone Formation of d-Gulomethylonic Acid

Time	$[\alpha]_D^{25}$	Time	$[\alpha]_D^{25}$
min.	*degrees*	*hrs.*	*degrees*
8	−5.2	20	−2.5
40	−2.7	48	−10.6
60	−0.9	96	−24.6
120	+1.8	288	−42.2
220	+4.0	20 (Days)	−47.9

of 12 gm. of silver oxide. The mixture was heated on the water bath for 8 hours at 80° with occasional stirring. The solution was then filtered and the filtrate concentrated under diminished pressure to 5 cc., whereupon a large quantity of crystalline material separated. After removal by filtration, the substance was recrystallized from water and dried over calcium chloride. The composition of the substance agreed with that of silver acetate.

13.800 mg. substance: 8.900 mg. Ag

$C_2H_3O_2Ag$.	Calculated.	Ag 64.6
166.88	Found.	" 64.4

Lactone Formation of d-Gulomethylonic Acid—*d*-Gulomethylonolactone (0.3005 gm.) was dissolved in exactly 5.0 cc. of 0.429 N

[9] Haworth, W. N., and Jones, D. I., *J. Chem. Soc.*, 2349 (1927).

sodium hydroxide solution and allowed to stand 1 hour at room temperature. The specific rotation, $[\alpha]_D^{25} = +9.7°$ (calculated as sodium gulomethylonate), remained constant at the end of this time. To 2.5 cc. of this solution there were then added 2.05 cc. of 0.498 N hydrochloric acid and the resulting solution was diluted to exactly 5.0 cc. The initial specific rotation was observed as soon as possible and the change in rotation observed at various time intervals, as shown in Table I.

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MAXIMUM ROTATIONS OF CARBOXYLIC ACIDS CONTAINING A PHENETHYL GROUP

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There was a twofold object in undertaking the present investigation. The first was to establish the maximum numerical values for the rotations of the configurationally related derivatives of disubstituted carboxylic acids containing a phenethyl group; the second, to extend the series of these acids beyond those previously described.

All the derivatives of the phenethyl series previously described were derived, directly from methylphenethylacetic (2-methyl-4-phenylbutyric) acid. The reduction of the ester of this acid to the corresponding carbinol, which was the parent substance of all other derivatives, may be expected to be accompanied by a certain degree of racemization. On the other hand, methylethylphenethylmethane (3-methyl-5-phenylpentane) can be prepared from active amyl alcohol whose maximum optical rotation is known. This reaction may be accomplished without racemization and, on the basis of the maximum rotation of this hydrocarbon, the maximum rotations of other members of the phenethyl series could be calculated. The hydrocarbon had been prepared by Klages and Sautter[1] and by Hardin[2] by the action of amylmagnesium iodide on benzaldehyde. The reaction was accompanied by a considerable degree of desaturation and therefore Klages found it expedient to desaturate the entire products of the reaction and then to reduce them by means of sodium and alcohol. These reactions are rather drastic in character and it seemed desirable to prepare the hydrocarbon by a set of milder reactions. Indeed, it was found that

[1] Klages, A., and Sautter, R., *Ber. chem. Ges.*, **37**, 649 (1904).

[2] Hardin, D., *J. chim. physiq.*, **6**, 584 (1908).

TABLE I

Maximum Rotations and Densities of Derivatives of 2-Phenethylpropionic Acid

CH_3—C(H)—$(CH_2)_2C_6H_5$	—COOH	—$COOC_2H_5$	—CH_2OH	—CH_2Br	—CH_2COOH	—$CH_2COOC_2H_5$	—CH_2CH_2OH	—CH_2CH_3
Density. . . .	1.0469 (20°)	0.9790 (28°)	0.9713 (20°)	1.2346 (20°)	1.0277 (23°)	0.9745 (20°)	0.9606 (20°)	0.8563 (25°)
$[\alpha]_D^{25}$.	+14.53	+12.33	−7.25	−2.41	−7.0	−4.50	−4.88	−5.52
$[M]_D^{25}$ based on parent acetic acid.	+51.20 (by resolution)	+50.29	−23.52	−10.81	−26.60	−19.63	−17.03	−17.72
$[M]_D^{25}$ based on hydrocarbon . . .			−46.60	−21.41	−52.69	−38.89	−33.72	−35.10

TABLE II

Maximum Molecular Rotation of Derivatives of H—C(CH_3)—$(CH_2)_2COOH$ with $(CH_2)_2C_6H_5$

Leading to H—C(CH_3)—$(CH_2)_3COOH$ with $(CH_2)_2C_6H_5$

	—Br	—COOH	—$COOC_2H_5$	—CH_2OH	—CH_2COOH
H—C(CH_3)—CH_2CH_2— with $(CH_2)_2C_6H_5$	−16.26	−6.52	−17.20	−20.07	−14.26
H—C(CH_3)—CH_2CH_2— with $(CH_2)_2C_6H_{11}$		−4.98			−6.31

when active amylmagnesium bromide was used for condensation with benzaldehyde, the reaction proceeded without desaturation. The resulting secondary carbinol was readily reduced with hydrogen iodide without heating. The hydrocarbon had, maximum $[M]_D^{25} = -35.10°$; D 25/4 = 0.8569; $n_D^{25} = 1.4862$. Klages and Sautter found $[M]_D^{14.5} = 25.8°$; D 19/4 = 0.8521; $n_D = 1.4896$.

The maximum rotations of all members of the phenethyl series, based on that of the hydrocarbon, are given in Tables I and II. Table I[3] contains the data on the substances previously described and Table II on those newly prepared.

The second task in regard to substances of the general type

$$\begin{array}{c} CH_3 \\ | \\ H—C—(CH_2)_nCOOH \\ | \\ CH_2CH_2C_6H_5 \end{array}$$

(where $n = 0$ or an integer) was to investigate the possible occurrence of a periodicity in the values of the optical rotation of these substances with successive increase in the value of n.

From Table III it may be seen that the periodic fluctuation of rotatory values of the four acids thus far prepared is unmistakable. Similar observations were made in the case of the analogous phenyl derivatives and in the carboxylic acids of the normal series. The underlying physical basis for this phenomenon will be the subject of a separate investigation.

EXPERIMENTAL

The derivatives of 2-methyl-4-phenylbutyric acid leading to the formation of 1-phenyl-3-methylpentane were prepared by the procedures described by Levene and Marker[4] in a previous paper. The details of their preparation will therefore be omitted here. The densities and the maximum values of their optical rotations are given in Table I. Only the resolution of the 2-methyl-4-

[3] In the article by Levene and Marker (*J. Biol. Chem.*, **110**, 312 (1935)), the second paragraph should read, "The relationship of (II) to (III) has now been established by the set of reactions given in Table I, which shows that dextro-2-*phenethyl*propionic acid (II) leads to levo-methylethyl*phenethyl*-methane (III)."

[4] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **110**, 311 (1935).

phenylbutyric acid will be given in detail, inasmuch as a higher value of the molecular rotation was attained than is reported by Levene and Marker.[4] The sequence of procedure in the preparation of the derivatives of higher molecular weights is indicated in Table II.

2-Methyl-4-Phenylbutyric Acid (β-Phenethylmethylacetic Acid)—This acid was prepared by the malonic synthesis and resolved by recrystallizing the cinchonidine salt from acetone as described by

TABLE III

Maximum Molecular Rotations of Carboxylic Acids Containing a Phenethyl or a Hexahydrophenethyl Group

CH_3 | H—C—COOH | $(CH_2)_2C_6H_5$ +51.2	CH_3 | H—C—CH_2COOH | $(CH_2)_2C_6H_5$ −52.69	CH_3 | H—C—CH_2CH_2COOH | $(CH_2)_2C_6H_5$ −6.52	CH_3 | H—C—$CH_2CH_2CH_2COOH$ | $(CH_2)_2C_6H_5$ −14.26
CH_3 | H—C—COOH | $(CH_2)_2C_6H_{11}$ +29.87	CH_3 | H—C—CH_2COOH | $(CH_2)_2C_6H_{11}$ −18.89	CH_3 | H—C—CH_2CH_2COOH | $(CH_2)_2C_6H_{11}$ −4.98	CH_3 | H—C—$CH_2CH_2CH_2COOH$ | $(CH_2)_2C_6H_{11}$ −6.31

Levene and Marker.[4] The maximum rotation observed after ten to twelve recrystallizations was

$$[\alpha]_D^{20} = \frac{-30.10^\circ}{1 \times 1.047} = -28.75^\circ;\ [M]_D^{20} = -51.20^\circ \text{ (homogeneous)}$$

1-Phenyl-3-methylpentane (methylethylphenethylmethane) was prepared by two procedures. (1) *5-Phenyl-3-methylpentanol-1* was treated with an excess of anhydrous liquid hydrogen iodide and allowed to stand for 1 week under pressure. It was then worked up in the usual way to remove the excess of hydrogen iodide. Without distillation of the iodide the product was reduced directly to the hydrocarbon by shaking it with Raney's catalyst in methyl

alcohol containing a slight excess of 10 per cent sodium hydroxide. Under a pressure of 3 atmospheres of hydrogen the reduction was completed in 1 hour. The catalyst was removed by centrifuging and washed with methyl alcohol. The methyl alcohol solution was thoroughly extracted with pentane. The extract was dried and evaporated, and the product distilled. B.p. 66–67° at 3 mm. or 96° at 13 to 14 mm. D 25/4 = 0.8563;[5] $n_D^{25} = 1.4860$.

$$[\alpha]_D^{25} = \frac{-4.73^\circ}{1 \times 0.856} = -5.52^\circ;\ \text{maximum}\ [M]_D^{25} = -17.72^\circ\ \text{(homogeneous)}$$

4 210 mg. substance: 13.705 mg. CO_2 and 4.210 mg. H_2O

$C_{12}H_{18}$.	Calculated.	C 88.8, H 11.2
162.1	Found.	" 88.8, " 11.2

(2) The hydrocarbon was also prepared by the action of active amylmagnesium bromide on benzaldehyde, according to the directions of Klages and Sautter[1] and of Hardin.[2]

25 gm. of active amyl bromide, $\alpha = -1.20^\circ$, were added dropwise to 5 gm. of magnesium in 100 cc. of anhydrous ether. The solution was refluxed for 5 minutes. Then 16 gm. (1 mole equivalent) of freshly distilled benzaldehyde were added dropwise without cooling. The carbinol was isolated as usual by hydrolysis and extraction with ether. The extract was washed with sodium carbonate solution, sodium bisulfite solution, and finally with water, and then dried over potassium carbonate. Yield 15 gm. B.p. 90° at 1 mm.; D 25/4 = 0.9628; $n_D^{25} = 1.5097$.

$$[\alpha]_D^{25} = \frac{-2.75^\circ}{1 \times 0.963} = -2.86^\circ;\ [M]_D^{25} = -5.09^\circ\ \text{(homogeneous)}$$

4.040 mg. substance: 12.030 mg. CO_2 and 3.600 mg. H_2O

$C_{12}H_{18}O$.	Calculated.	C 80.6, H 10.1
178.1	Found.	" 81.1, " 10.0

This carbinol was sealed in a bomb tube with anhydrous liquid hydrogen iodide and allowed to stand overnight in a solid carbon dioxide-chloroform bath. It was then allowed to stand for 48 hours at room temperature. The tube was now cooled and opened and the hydrogen iodide allowed to escape slowly. The residue was poured onto ice and extracted with pentane, and the extract was shaken with potassium iodide solution until nearly colorless.

[5] All densities reported in this paper are corrected *in vacuo*.

Finally, it was shaken with 50 per cent calcium chloride solution and dried over anhydrous calcium chloride.

After removal of the pentane the residue was distilled at 0.8 mm., the main fraction boiling at 60°.

$$[\alpha]_D^{25} = \frac{-3.50°}{1 \times 0.857} = -4.09°;\ [M]_D^{25} = -6.62° \text{ (homogeneous)}$$

The active amyl bromide used as starting material had a molecular rotation of −1.49°. The maximum molecular rotation found by Levene and Marker[6] for the bromide is −7.9°. Hence the maximum rotation of this hydrocarbon is

$$[M]_D^{25} = \frac{-6.62° \times 7.9}{1.49} = -35.1° \text{ (homogeneous)}$$

$$D\,25/4 = 0.8569°;\ n_D^{25} = 1.4862$$

3.050 mg. substance: 9.920 mg. CO_2 and 3.020 mg. H_2O

$C_{12}H_{18}$.	Calculated.	C 88.8, H 11.2
162.1	Found.	" 88.7, " 11.1

The identity of these two specimens of hydrocarbon is shown by the good agreement of their densities and refractive indices. The maximum molecular rotation of this hydrocarbon was used as the basis for calculating the maximum molecular rotations of the rest of the phenethylmethyl derivatives. The figures in the last line of Table I were therefore obtained by multiplying the figures in the preceding line by the factor 1.981, which is the relation of the rotations of the two samples of 1-phenyl-3-methylpentane obtained from the two different sources.

Active 4-Methyl-6-Phenylhexanoic Acid (Active 4-β-Phenethyl-4-Methylbutyric Acid)—This acid was made by two different sets of reactions.

(1) Active 1-phenyl-3-methyl-5-bromopentane, $[\alpha]_D^{25} = -1.72°$, was converted into its Grignard reagent in the usual manner. This was then cooled with an ice-salt mixture while a slow stream of carbon dioxide was passed over the stirred reaction mixture until the RMgX compound had been destroyed. The mixture was poured onto ice and hydrochloric acid and the organic acid extracted with ether. The ether solution was washed with sodium carbonate solution from which the organic acid was recovered on

[6] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **103**, 299 (1933).

acidification. It was dried with sodium sulfate and distilled. B.p. 152–154° at 1 mm. The yield was 58.5 per cent of the theoretical, based on the amount of bromide used.

(2) 235 gm. (1.05 moles) of 1-phenyl-3-methyl-4-bromobutane, $\alpha_D^{25} = -5.62°$ (obtained from the acetic acid derivative having $\alpha_D^{25} = +28.5°$), were condensed with 1.0 mole of malonic ester in absolute alcohol to which 0.9 mole of sodium had been added. After 4 hours refluxing, the solution was neutral to litmus. The yield of ester was 222 gm. (0.73 mole), or 81 per cent yield based on the amount of sodium used.

The ester was hydrolyzed by refluxing overnight with 3 moles of alcoholic potassium hydroxide. The organic acid was obtained on acidification and was crystallized from benzene by adding petroleum ether (b.p. 60–70°) until the solution was turbid. M.p. 96–98°. Yield 152 gm. (0.61 mole) or 83.5 per cent of the theoretical.

This acid decomposed at 180° and the resulting acid was distilled. B.p. 162° at 1 mm. Yield 122 gm. (0.595 mole) or 97.5 per cent from the malonic acid. D 21.5/4 = 1.018; $n_D^{25} = 1.5066$.

$$[\alpha]_D^{21.5} = \frac{-1.54°}{1 \times 1.018} = -1.51°; \text{maximum } [\alpha]_D^{21.5} = -1.51° \times 1.056 = 1.60°$$

(based on parent acetic acid)

$[M]_D^{21.5} = -3.29°$ (homogeneous) (based on parent acetic acid)

Maximum $[M]_D^{21.5} = -3.29° \times 1.981 = -6.52°$ (based on hydrocarbon)

3.715 mg. substance: 10.315 mg. CO_2 and 2.980 mg. H_2O

$C_{13}H_{18}O_2$.	Calculated.	C 75.6, H 8.8
206.1	Found.	" 75.7, " 9.0

Ethyl Ester of 6-Phenyl-4-Methylhexanoic Acid (Ethyl Ester of 4-β-Phenethyl-4-Methylbutyric Acid)—The ethyl ester of this acid was prepared in the usual way with absolute ethyl alcohol, with sulfuric acid as a catalyst, a 93.5 per cent yield being obtained. B.p. 122° at 2 mm. D 25/4 = 0.965; $n_D^{25} = 1.4872$.

$$[\alpha]_D^{25} = \frac{-3.39°}{1 \times 0.9650} = -3.51°; \text{maximum } [\alpha]_D^{25} = -3.51° \times 1.056 = -3.71°$$

(based on parent acetic acid)

$[M]_D^{25} = -8.69°$ (homogeneous) (based on parent acetic acid)

Maximum $[M]_D^{25} = -8.69° \times 1.981 = -17.20°$ (based on parent acetic acid)

4.460 mg. substance: 12.560 mg. CO_2 and 3.800 mg. H_2O

$C_{15}H_{22}O_2$.	Calculated.	C 76.9, H 9.5
234.1	Found.	" 76.8, " 9.5

4-Methyl-6-Phenylhexanol-1 (*4-Phenethyl-4-Methylbutanol-1*)—The above ester was reduced in the usual way by adding it in absolute alcohol solution to an emulsion of sodium in toluene. Yield 92.5 per cent. B.p. 155° at 10 mm. *D* 25/4 = 0.9477; $n_D^{25} = 1.5080$.

$$[\alpha]_D^{25} = \frac{-4.73°}{1 \times 0.9477} = -4.99° \text{ (homogeneous)}$$

Maximum $[\alpha]_D^{25} = -4.99° \times 1.056 = -5.27°$ (based on parent acetic acid)

$[M]_D^{25} = -10.13°$ (based on parent acetic acid)

Maximum $[M]_D^{25} = -10.13° \times 1.981 = -20.07°$ (based on hydrocarbon)

4.791 mg. substance: 14.270 mg. CO_2 and 4.475 mg. H_2O

$C_{13}H_{20}O$.	Calculated.	C 81.1, H 10.5
192.2	Found.	" 81.2, " 10.5

5-Methyl-7-Phenylheptanoic Acid (*5-β-Phenethyl-5-Methylvaleric Acid*)—This acid was prepared by the action of carbon dioxide on 4-methyl-6-phenylhexylmagnesium bromide, the yield being 73 per cent. B.p. 172° at 2.5 mm. *D* 25/4 = 1.0014; $n_D^{25} = 1.5039$.

$$[\alpha]_D^{25} = \frac{-3.10°}{1 \times 1.0014} = -3.10° \text{ (homogeneous)}$$

Maximum $[\alpha]_D^{25} = -3.10° \times 1.053 = -3.27°$ (based on parent acetic acid)

$[M]_D^{25} = -7.20°$ (based on parent acetic acid)

Maximum $[M]_D^{25} = -7.20° \times 1.981 = -14.26°$ (based on hydrocarbon)

5.250 mg. substance; 14.700 mg. CO_2 and 4.210 mg. H_2O

$C_{14}H_{20}O_2$.	Calculated.	C 76.3, H 9.2
220.2	Found.	" 76.4, " 9.0

4-Methyl-6-Cyclohexylhexanoic Acid—This acid was prepared by reducing the corresponding phenyl derivative in glacial acetic acid with hydrogen, with platinum oxide as catalyst. B.p. 180° at 10 mm. *D* 23/4 = 0.9528; $n_D^{25} = 1.4657$.

$$[\alpha]_D^{23} = \frac{-0.57°}{1 \times 0.9528} = -0.60° \text{ (homogeneous)}$$

Maximum $[\alpha]_D^{23} = -0.60° \times 1.979 = -1.18°$ (based on parent acetic acid)

$[M]_D^{23} = -2.51°$ (based on parent acetic acid)

Maximum $[M]_D^{23} = -2.51° \times 1.981 = -4.98°$ (based on hydrocarbon)

3.950 mg. substance: 10.665 mg. CO_2 and 4.040 mg. H_2O

$C_{13}H_{24}O_2$.	Calculated.	C 73.5, H 11.4
212.2	Found.	" 73.6, " 11.4

5-Methyl-7-Cyclohexylheptanoic Acid—5-Methyl-7-phenylheptanoic acid was reduced in glacial acetic acid with platinum oxide

as a catalyst. It was isolated by distillation. B.p. 136–140° at 0.3 mm. D 25/4 = 0.9455; n_D^{25} = 1.4658.

$$[\alpha]_D^{25} = \frac{-1.27°}{1 \times 0.9455} = -1.34° \text{ (homogeneous)}$$

Maximum $[\alpha]_D^{25} = -1.34° \times 1.056 = -1.41°$ (based on parent acetic acid)

$[M]_D^{25} = -3.18°$ (based on parent acetic acid)

Maximum $[M]_D^{25} = -3.18° \times 1.981 = -6.31°$ (based on hydrocarbon)

0.1041 gm. substance required 4.62 cc. 0.1 N NaOH; mol. wt. 225.3

5.154 mg. " : 14.024 mg. CO_2 and 5.344 mg. H_2O

$C_{14}H_{26}O_2$.	Calculated.	C 74.3, H 11.6
226.2	Found.	" 74.2, " 11.6

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OPTICAL ROTATION OF METHYLOCTYLPHENETHYL-METHANE

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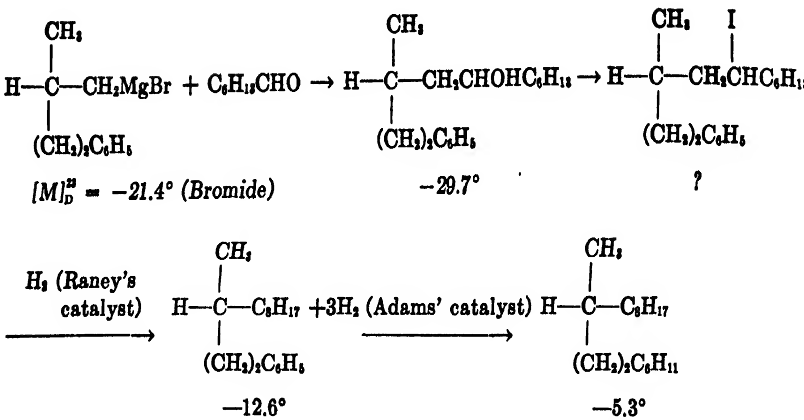
In the case of secondary carbinols, the cyclohexyl group resembles the isopropyl group in its effect on the direction of optical rotation. Thus in the two homologous series, starting with methylisopropylcarbinol and methylcyclohexylcarbinol respectively, the rotation of the first member is in a direction opposite from that of the higher members.[1] Furthermore, in the series of carbinols of the type

$$\begin{array}{c} CH_3 \\ | \\ H—C—OH \\ | \\ (CH_2)_nR \end{array}$$

(where n = 0 or an integer and R = an isopropyl or cyclohexyl group) both the isopropyl and the cyclohexyl groups have the same effect on the direction of rotation as the corresponding propyl and hexyl groups. In fact, in the case where n = 2, the isopropyl group resembles the normal propyl group even in its quantitative effect on the optical rotation, so that the maximum rotation of the methylamylisoamylmethane is in the neighborhood of $[M]_D^{20} = 0.2°$.[2] It was therefore desirable to investigate the effect on the rotation of the distance from the asymmetric center as of the cyclohexyl group compared with the effect of the isopropyl group. With this object in view the hexahydrophenethyloctylmethylmethane was prepared by the accompanying set of reactions.

[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **97**, 379 (1932).
[2] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **95**, 1 (1932).



The rotation of the intermediates of the final hydrocarbon are given in Table I. From these results it may be seen that the cyclohexyl group furnishes a much higher partial rotation than does the isopropyl group.

EXPERIMENTAL[3]

1-Phenyl-3-Methylundecanol-5

$$C_6H_5CH_2CH_2\overset{CH_3}{\overset{|}{C}}HCH_2\overset{OH}{\overset{|}{C}}HC_6H_{13}$$

The Grignard reagent was made from 37 gm. (0.163 mole) of phenethylmethylethyl bromide (1-bromo-2-methyl-4-phenylbutane) having a specific rotation of $-2.41°$. Heptaldehyde was added in ether solution until the RMgX had disappeared as shown by the color test. This required 15 gm. (0.132 mole) of heptaldehyde. The carbinol was obtained by the usual procedure. The yield was 27 gm. or 78 per cent of the theoretical, based on the amount of aldehyde used. B.p. 182–184° at 5 mm. pressure. D 20/4 = 0.9134 (*in vacuo*); $n_D^{25} = 1.4952$.

$$[\alpha]_D^{20} = \frac{-2.64°}{1 \times 0.913} = -2.89° \text{ (homogeneous)}$$

[3] The preparation of starting materials and the derivation of the maximum rotations given in Table I are published in the preceding paper by the same authors.

The maximum and molecular rotations are given in Table I.

3.400 mg. substance: 10.280 mg. CO_2 and 3.500 mg. H_2O
$C_{18}H_{30}O$. Calculated, C 82.4, H 11.5; found, C 82.3, H 11.5

Phenethylmethyloctylmethane (1-Phenyl-3-Methylundecane)

$$C_6H_5CH_2CH_2\overset{\displaystyle CH_3}{\overset{|}{C}}HC_8H_{17}$$

The carbinol was converted to the iodide by allowing it to stand for 2 days under pressure with pure hydrogen iodide. The iodide

TABLE I

Maximum Rotation of Derivatives Leading to Methylhexahydrophenethylmethane

Compounds	α	Maximum [M] based on hydrocarbon
	degrees	*degrees*
$C_6H_5CH_2CH_2—\overset{\displaystyle CH_3}{\overset{\vert}{C}}H—CH_2—\underset{\displaystyle H}{\underset{\vert}{\overset{\displaystyle OH}{\overset{\vert}{C}}}}—C_6H_{13}$	−2.64	−29.68
$C_6H_5CH_2CH_2\overset{\displaystyle CH_3}{\overset{\vert}{C}}H—C_8H_{17}$	−1.12	−12.59
$C_6H_{11}CH_2CH_2\overset{\displaystyle CH_3}{\overset{\vert}{C}}H—C_8H_{17}$	−0.44	−5.27

was washed with water and extracted with ether in the usual way. Distillation of the iodide was attempted, but there was too much decomposition, so it was reduced directly with Raney's catalyst. The iodide was dissolved in petroleum ether, b.p. 30–40°, and added to methyl alcohol containing an excess of 10 per cent sodium hydroxide. The non-homogeneous mixture (total volume about 50 cc.) was shaken in the presence of hydrogen at atmospheric pressure with a large excess of Raney's catalyst. The reduction proceeded smoothly until the required amount of hydrogen had been absorbed (2 to 3 hours). The catalyst was removed and washed by centrifuging. The methyl alcohol solution was ex-

tracted with pentane. The solution was washed with water and then with 30 per cent alkali, dried over calcium chloride, and distilled. B.p. 143–145° at 2 mm. pressure. *D* 23.5/4 = 0.8590 (*in vacuo*); $n_D^{24.5}$ = 1.4852.

$$[\alpha]_D^{23.5} = \frac{-1.12°}{1 \times 0.859} = -1.31° \text{ (homogeneous)}$$

3.700 mg. substance: 11.885 mg. CO_2 and 4.120 mg. H_2O
$C_{18}H_{30}$. Calculated, C 87.7, H 12.3; found, C 87.6, H 12.5

1-Cyclohexyl-3-Methylundecane $C_6H_{11}CH_2CH_2\overset{\displaystyle CH_3}{\overset{|}{C}}HC_8H_{17}$

This compound was prepared by hydrogenation of the preceding phenyl derivative in an acetic acid-ethyl acetate solution, Adams' platinum oxide catalyst being used. B.p. 147° at 3 mm. pressure. *D* 20/4 = 0.8260 (*in vacuo*); $n_D^{24.6}$ = 1.4553.

$$[\alpha]_D^{20} = \frac{-0.44°}{1 \times 0.826} = -0.53° \text{ (homogeneous)}$$

2.960 mg. substance: 9.300 mg. CO_2 and 3.795 mg. H_2O
$C_{18}H_{36}$. Calculated, C 85.6, H 14.4; found, C 85.7, H 14.3

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CONFIGURATIONAL RELATIONSHIPS OF METHYL-PHENYL- AND METHYLHEXYLACETIC ACIDS AND AN ATTEMPT AT THE CORRELATION OF THE CONFIGURATIONS OF 2-HYDROXY ACIDS WITH THOSE OF DISUBSTITUTED ACETIC ACIDS CONTAINING A METHYL GROUP

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In the series of acids of the general type

$$\begin{array}{c} CH_3 \\ | \\ H—C—COOH \\ | \\ (CH_2)_n \\ | \\ C_6H_5 \end{array}$$

($n = 0$ or an integer) the configurational relationships of the acids with $n = 1$ and 2 have already been established.[1–3] In order to elucidate the effect on the rotations of the acids of this type of the distance of the C_6H_5 group from the asymmetric center, there remained to correlate the configuration of the acid with $n = 0$ to the above two acids.

Inasmuch as the configurations of methylphenylacetic acid to methylethylcyclohexylmethane had already been established, there remained to correlate the configuration of this hydrocarbon to that of methylethylacetic acid. The principal set of reactions by which this task was accomplished is given in Table I.

From the fact that levo-methylethylcyclohexylmethane (IV) is correlated to dextro-methylethylacetic acid (I) and dextro-methylethylhexylmethane (VI), it follows that dextro-methylcyclohexylacetic acid (VII) is correlated to dextro-methylhexylacetic acid (II) and to levo-methylethylhexylmethane (III). It therefore

[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **110**, 299, 311 (1935).
[2] Levene, P. A., *J. Biol. Chem.*, **110**, 323 (1935).
[3] Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **111**, 725 (1935).

follows on the basis of the observations of Levene and Marker,[1] of Levene,[2] and of Levene and Harris[3] that the configurations of

TABLE I

Set of Reactions Leading to Correlation of Configuration of Derivatives Containing a Hexyl, Cyclohexyl, or Phenyl Group

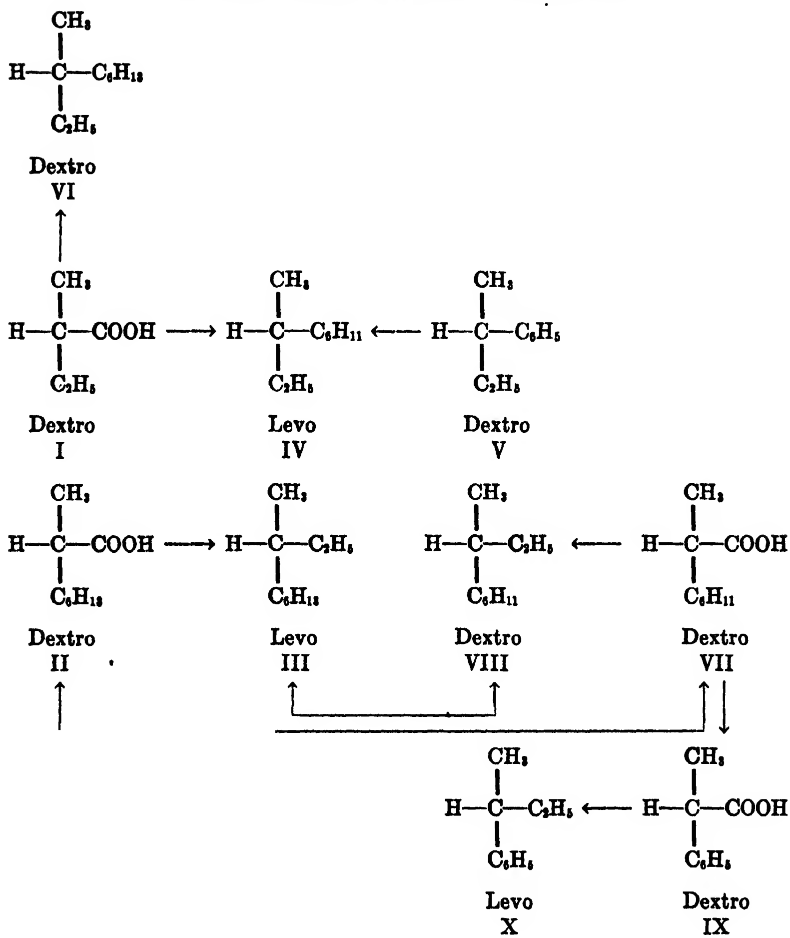


TABLE II

Maximum $[M]_D^{25}$ of Configurationally Related Hydrocarbons and Acids

CH_3 H—C—COOH C_6H_5	CH_3 H—C—C_2H_5 C_6H_5	CH_3 H—C—COOH CH_2 C_6H_5	CH_3 H—C—C_2H_5 CH_2 C_6H_5	CH_3 H—C—COOH $(CH_2)_2$ C_6H_5	CH_3 H—C—C_2H_5 $(CH_2)_2$ C_6H_5
+112°	−36.5°	+31°	−9.25°	+51.2°	−35.10°
CH_3 H—C—COOH C_6H_{13}	CH_3 H—C—C_2H_5 C_6H_{13}	CH_3 H—C—COOH C_7H_{15}	CH_3 H—C—C_2H_5 C_7H_{15}	CH_3 H—C—COOH C_8H_{17}	CH_3 H—C—C_2H_5 C_8H_{17}
About +26.0°	−12.5°	+27.3°	About −12.5°	About +28°	−17.4°
CH_3 H—C—COOH C_6H_{11}	CH_3 H—C—C_2H_5 C_6H_{11}	CH_3 H—C—COOH CH_2 C_6H_{11}	CH_3 H—C—C_2H_5 CH_2 C_6H_{11}	CH_3 H—C—COOH $(CH_2)_2$ C_6H_{11}	CH_3 H—C—C_2H_5 $(CH_2)_2$ C_6H_{11}
+25.8°	+19.5°	+28.4°	−19.3°	+29.87°	−16.8°

the acids containing a phenyl or a cyclohexyl group are correlated on the one hand among themselves and the other hand to the corresponding normal acids, as given in Table II.

Effect of the Distance from the Asymmetric Center of Phenyl and Cyclohexyl Groups on Rotations of the Substituted Acetic Acids

Case of the Phenyl Group—It may be seen from Table II that the substitution of the *n*-hexyl group by a phenyl enhances the rotation to a very high degree. In the case of the substitution of an *n*-heptyl group by a benzyl group, the rotation is only slightly enhanced. Thus the removal of the C_6H_5 group at a distance of one CH_2 group seems to have little effect on the rotation, but the substitution of the normal octyl group by a phenethyl again brings about an increase of the rotation in the same direction. Thus there seems to be an alternation in the shift of the rotation of the acids of the first row of Table II with the successive increase of the distance of C_6H_5 from the asymmetric center.

Case of the Cyclohexyl Group—In this case no marked difference is observed between the rotation of the normal and cyclic series. No periodicity is detected in either of the two series.

Attempt to Correlate α-Hydroxy Acids with Corresponding Normal Disubstituted Acetic Acids—The question arises whether the knowledge of the configurations of the hydrocarbons given in Table II may serve for the correlation of acids of the type

$$\begin{array}{c} OH \\ | \\ H—C—COOH \\ | \\ R \end{array} \quad \text{to acids of the type} \quad \begin{array}{c} CH_3 \\ | \\ H—C—COOH \\ | \\ R \end{array}$$

(R = a normal alkyl group, phenyl, benzyl, phenethyl, and their hydrogenated groups).

This task can be accomplished if it is admitted with Boys[4] that for simple substances such as

$$\begin{array}{c} CH_3 \\ | \\ H—C—C_2H_5 \\ | \\ R \end{array} \quad \text{or} \quad \begin{array}{c} OH \\ | \\ H—C—C_2H_5 \\ | \\ R \end{array}$$

($R = C_3H_7$ or a higher homologous radicle) the absolute configuration is determined by clockwise or counter-clockwise arrangement of the radicles according to their increasing volume ($H < OH < CH_3 < C_2H_5 < C_nH_{2n+1}$) ($n = 3$ or a higher integer). This assumption was borne out by the experimental observations of Levene and Haller[5] and Levene and Marker.[6] Boys defines such spatial configurations as dextrorotatory which, viewed from the group with highest volume towards the observer, have the remaining three groups in clockwise order according to the descending volumes of the groups $A < B < C < D$.

In projection the dextrorotatory hydrocarbons may be represented in such a way that groups are arranged clockwise in descending order of volume, the groups A and C situated in the plane of the paper and the groups B and D in a plane above the paper. The plane of the paper is represented by the lighter line as $\begin{array}{c} B \\ C —|— A. \\ | \\ D \end{array}$

On the basis of this assumption the above hydrocarbon and carbinol are considered levorotatory. It would then follow that

$$\begin{array}{c} OH \\ | \\ H—C—COOH \\ | \\ R \\ \\ \text{Dextro} \end{array} \quad \text{and} \quad \begin{array}{c} CH_3 \\ | \\ H—C—COOH \\ | \\ R \\ \\ \text{Dextro} \end{array}$$

(R = as above for both substances) are configurationally related.

[4] Boys, S. F., *Proc. Roy. Soc. London, Series A*, **144**, 655 (1934).
[5] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **79**, 475 (1928).
[6] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 761 (1931).

TABLE III—*Maximum Molecular Rotations of Configurationally Related Secondary Carbinols and Trisubstituted Methanes*

$H-C(OH)(C_2H_5)-C_6H_{13}$	$H-C(OH)(C_2H_5)-C_6H_{11}$	$H-C(OH)(C_2H_5)-C_6H_5$	$H-C(OH)(C_2H_5)-CH_2-C_6H_{13}$	$H-C(OH)(C_2H_5)-CH_2-C_6H_{11}$	$H-C(OH)(C_2H_5)-CH_2-C_6H_5$	$H-C(OH)(C_2H_5)-CH_2-CH_2-C_6H_{13}$	$H-C(OH)(C_2H_5)-CH_2-CH_2-C_6H_{11}$	$H-C(OH)(C_2H_5)-CH_2-CH_2-C_6H_5$
−11.6°	+11.5°	+39.7°	−10.6°	−41.0°	−51.7°	−10.7°	−10.0°	−31.0°
$H-C(CH_3)(C_2H_5)-C_6H_{13}$	$H-C(CH_3)(C_2H_5)-C_6H_{11}$	$H-C(CH_3)(C_2H_5)-C_6H_5$	$H-C(CH_3)(C_2H_5)-CH_2-C_6H_{13}$	$H-C(CH_3)(C_2H_5)-CH_2-C_6H_{11}$	$H-C(CH_3)(C_2H_5)-CH_2-C_6H_5$	$H-C(CH_3)(C_2H_5)-CH_2-CH_2-C_6H_{13}$	$H-C(CH_3)(C_2H_5)-CH_2-CH_2-C_6H_{11}$	$H-C(CH_3)(C_2H_5)-CH_2-CH_2-C_6H_5$
−12.5°	+19.5°	−36.5°	−12.5°(App.)	−19.3°	−9.25°	−17.4°	−16.8°	−35.10°

Correlation of Phenylated Secondary Carbinols of the Type

$$H-C(OH)(CH_3)-(CH_2)_n-R \quad \textit{to the Hydrocarbons of the Type} \quad H-C(CH_3)(C_2H_5)-(CH_2)_n-R$$

In the above $n = 0$ or an integer, R = a phenyl or cyclohexyl group.

Inasmuch as the carbinols of the above type with $n = 0$, 1, and 2 have been previously correlated among themselves and every one to the corresponding normal secondary carbinol,[7] all the carbinols given in the first row of Table III may be correlated to the hydrocarbons of the above type as given in Table III.

If the validity of the above conclusion is admitted, it is possible to speculate on the effect on rotation of substitution of a hexyl group by a phenyl or by a cyclohexyl in the case of the carbinols of the above type and of the corresponding hydrocarbons.

From Table III it may be seen that the substitution of C_6H_{13} by $—C_6H_{11}$ when $n = 0$ or 1 has a similar effect on the rotation in the case of the carbinols and in that of the hydrocarbons; the effect of substitution by C_6H_5 is in the opposite sense in the case of the carbinols as compared with that of the hydrocarbons.

When $n = 2$ the substitution of C_6H_{13} by C_6H_{11} has little effect on the molecular rotation of the substances; substitution by C_6H_5 produces an identical effect in the case of the carbinol and in that of the hydrocarbon.

SUMMARY

1. The similarity of the configurations of the hydrocarbons I and II

[7] Levene, P. A., and Stevens, P. G., *J. Biol. Chem.*, **89**, 471 (1930); **87**, 375 (1930). Levene, P. A., and Walti, A., *J. Biol. Chem.*, **94**, 367 (1931). Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **97**, 379 (1932).

$$\begin{array}{ccc} & CH_3 & \\ & | & \\ H— & C & —C_6H_{11} \\ & | & \\ & C_2H_5 & \end{array} \qquad\qquad \begin{array}{ccc} & CH_3 & \\ & | & \\ H— & C & —C_6H_{13} \\ & | & \\ & C_2H_5 & \end{array}$$

Levo — Dextro

I — II

has been established by direct chemical methods.

2. This correlation permitted (*a*) the correlation of the configurations of hydrocarbons of the general type

$$\begin{array}{ccc} & CH_3 & \\ & | & \\ H— & C & —C_2H_5 \\ & | & \\ & (CH_2)_n & \\ & | & \\ & R & \end{array}$$

(n = 0, 1, or 2; R = C_6H_{13}, C_6H_{11}, and C_6H_5), (*b*) the correlation of the configurations of acids of the general type

$$\begin{array}{ccc} & CH_3 & \\ & | & \\ H— & C & —COOH \\ & | & \\ & (CH_2)_n & \\ & | & \\ & R & \end{array}$$

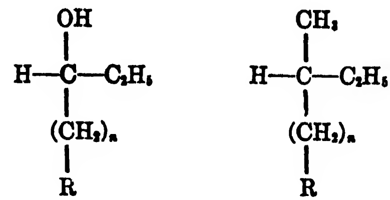
(n and R = as above), and thus to follow the effect on the rotation of the distance from the asymmetric carbon atom of the phenyl and cyclohexyl groups on the rotation of these acids.

3. An attempt was made to correlate the configurations of acids of the general type

$$\begin{array}{ccc} & OH & \\ & | & \\ H— & C & —COOH \\ & | & \\ & R & \end{array} \qquad \text{with those of} \qquad \begin{array}{ccc} & CH_3 & \\ & | & \\ H— & C & —COOH \\ & | & \\ & R & \end{array}$$

(R = a normal aliphatic radicle).

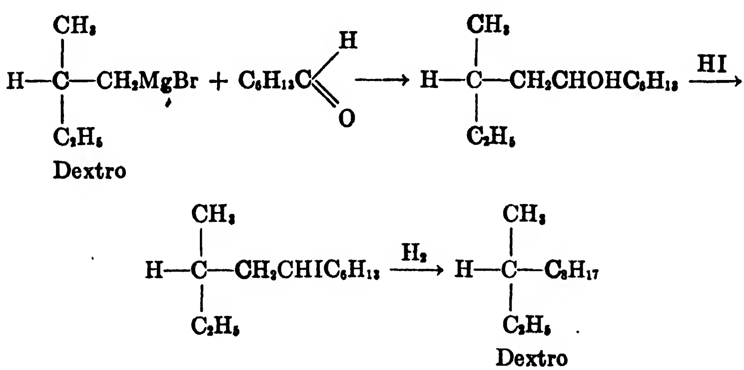
4. An attempt was made to correlate secondary carbinols and hydrocarbons of the types given below.



(n = 0, 1, or 2; R = C_6H_{13}, C_6H_{11}, and C_6H_5).

Synthesis of the Optically Active Methylethylcyclohexylmethane and Its Correlation to Methylethyloctylmethane—The configurations of the two hydrocarbons were correlated through their syntheses.

The *aliphatic hydrocarbon* was prepared by the following set of reactions.



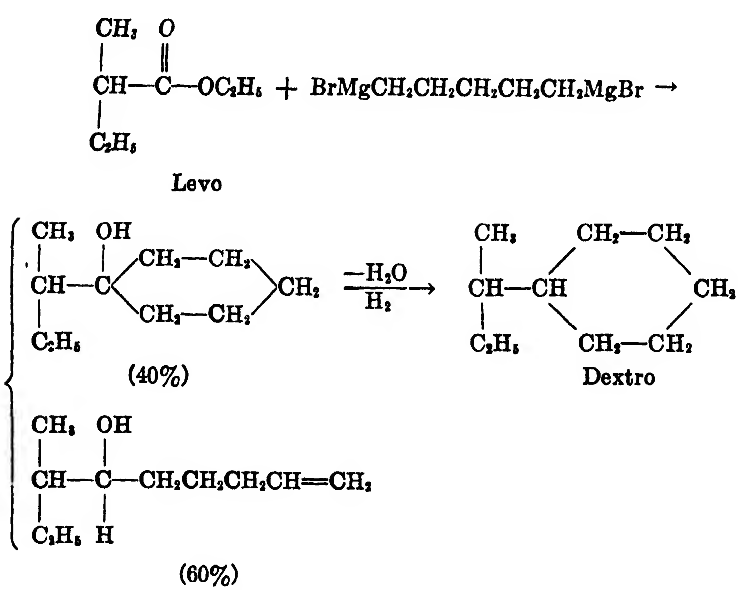
The *methylethylcyclohexylmethane* was prepared in the following way. Grignard and Vignon[8] describe the preparation of 1-methylcyclohexanol-1 by adding ethyl acetate to pentamethylenedimagnesium bromide. Likewise it should be possible to obtain active 1-sec.-butylcyclohexanol-1 by treating the same Grignard reagent with the active ethyl ester of methylethylacetic acid. When the reaction was carried out, a carbinol having the correct composition was obtained, but on dehydration and reduction of the unsaturated compound an active hydrocarbon was obtained which did not have the correct physical properties as given by Signaigo

[8] Grignard, V., and Vignon, G., *Compt. rend. Acad.*, **144**, 1358 (1907).

and Cramer[9] and by Levene and Marker.[10] On examination of the physical properties of the carbinol and of the unsaturated hydrocarbon it was found that they did not correspond to those of homologous or isomeric compounds described by Signaigo and Cramer.[9]

It was then discovered that the reaction product contained a highly unsaturated compound. The product absorbed 60 per cent of the theoretical amount of bromine, calculated on the basis of the carbinol. On distillation the forerun spontaneously lost water and proved to be a mixture of active sec.-butylcyclohexene and active sec.-butylcyclohexanol. From it the active sec.-butylcylclohexane was prepared as described in the experimental part.

The high boiling dibromide mentioned above had a composition of a non-cyclic dibromo secondary carbinol $C_{10}H_{20}OBr_2$. The structure of the unsaturated carbinol and of its dibromide was not further investigated. The course of the reaction may be formulated in the following way.



[9] Signaigo, F. K., and Cramer, P. L., *J. Am. Chem. Soc.*, **55**, 3326 (1933).
[10] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **97**, 563 (1932).

EXPERIMENTAL

Levo-1-Sec.-Butylcyclohexanol-1—Pentamethylene bromide was prepared as described in "Organic syntheses,"[11] except that it was found necessary to shake a pentane solution of the bromide with cold concentrated sulfuric acid to remove all of the benzonitrile.

The reaction between this dibromide and magnesium in ether solutions was easily started by adding a few crystals of iodine. 90 gm. (0.39 mole) of pentamethylene bromide in ether solution was added dropwise to an excess of magnesium (20 gm.; 0.83 mole) which was covered by ether. The total volume at the end of the reaction was about 500 cc. To this non-homogeneous Grignard reagent was added active ethyl ethylmethylacetate, having

$$[\alpha]_D^{22} = \frac{-6.18^\circ}{0.870} = -7.11^\circ$$

until the RMgX was completely destroyed. The ethyl ester was prepared in the usual manner from active ethylmethylacetic acid

$$[\alpha]_D^{20} = \frac{-7.81^\circ}{0.934} = -8.36^\circ$$

which was resolved as described by Schutz and Marckwald.[12]

The carbinol was obtained by first hydrolyzing the reaction mixture with aqueous ammonium chloride solution and then extracting with ether which was washed with water and dried over sodium sulfate. The carbinol boiled constantly at 99° at 14 to 16 mm. pressure. Yield 45 gm. (0.288 mole) or 74 per cent of the theoretical. $\alpha_D^{25} = +3.96°$.

This carbinol was treated in the following manner. 19.5 gm. (0.125 mole) of the carbinol were dissolved in chloroform and cooled in an ice bath. 20 gm. of bromine (0.125 mole) were dissolved in chloroform and made up roughly to a volume of 50 cc. This was added to the cooled carbinol solution until the red bromine color persisted. Exactly 30 cc., or 60 per cent, of the theoretical amount of bromine were used. This solution was shaken, first with sodium bisulfite solution, then water, and finally sodium bicarbonate solution. It was dried over sodium sulfate and distilled under reduced pressure through a short

[11] Gilman, H., Organic syntheses, New York, coll. 1, 419 (1932).
[12] Schutz, O., and Marckwald, W., *Ber. chem. Ges.*, 29, 52 (1896).

column. After the chloroform was removed, the low boiling material from the bromide was separated by distillation at 5 mm. pressure, with a bath cooled with solid carbon dioxide for a condenser. This distillate was worked up for the cyclohexyl derivatives as described below.

The high boiling fraction distilled at 161° at 5 mm. pressure. The analysis corresponded to the theoretical for $C_{10}H_{20}OBr_2$.

4.456 mg. substance: 6.275 mg. CO_2 and 2.500 mg. H_2O
3.690 " " : 4.390 " AgBr

$C_{10}H_{20}OBr_2$. Calculated.	C 38.0, H 6.35, Br 50.6	
Found.	" 38.4, " 6.27, " 50.6	

Levo-1-Sec.-Butylcyclohexene-1—The low boiling distillate obtained in the previous experiment was wet and was also unsaturated, indicating spontaneous desaturation on distillation. Without attempting to isolate the carbinol, the material was distilled at atmospheric pressure. Water was given off above 140° and the hydrocarbon came over between 170–178°. The hydrocarbon was taken up in petroleum ether, dried with calcium sulfate, and redistilled. The substance was slightly colored and turbid, so it was redistilled from sodium. Yield 5 gm. B.p. 172–174° uncorrected. *D* 20/20 = 0.829 (*in vacuo*). $n_D^{20} = 1.4590$.

$$[\alpha]_D^{20} = \frac{-3.36°}{0.829} = -4.06°$$

3.780 mg. substance: 12.060 mg. CO_2 and 4.400 mg. H_2O

$C_{10}H_{18}$. Calculated.	C 87.0, H 13.0
Found.	" 87.0, " 13.0

Dextro-2-Cyclohexylbutane—The sec.-butylcyclohexene which had not been distilled from sodium was dissolved in 20 cc. of absolute alcohol and reduced with hydrogen and 0.1 gm. of PtO_2 as a catalyst. Reduction was complete in 3 to 5 minutes. The solution was then diluted with 10 volumes of water, centrifuged to remove the catalyst, and extracted with petroleum ether. This was dried over calcium chloride and then distilled. After redistillation from sodium the following constants were obtained. B.p. 176–178° at 760 mm. *D* 20/20 = 0.815 (*in vacuo*). $n_D^{20} = 1.4460$.

$$[\alpha]_D^{20} = \frac{+0.48°}{0.815} = +0.59°$$

3.870 mg. substance: 12.125 mg. CO_2 and 4.970 mg. H_2O

$C_{10}H_{20}$. Calculated.	C 85.7, H 14.3
Found.	" 85.4, " 14.4

3-Methylundecanol-5—26 gm. of active amyl bromide, $\alpha_D^{25} = +2.40°$ (homogeneous), were added to 5.5 gm. of magnesium turnings in 100 cc. of dry ether. After all the bromide was added, the mixture was refluxed for 10 minutes. Then 21 gm. of freshly distilled heptaldehyde dissolved in 35 cc. of dry ether were added without cooling. The reaction mixture was poured onto ice, ammonium chloride solution added, and the ether layer separated. The extracts were washed with ammonium chloride solution, water, and dilute carbonate, and then dried over anhydrous potassium carbonate, and distilled. B.p. 70–75° at 0.1 mm. pressure; also 113° at 12 mm. pressure. Yield 14 gm. *D* 24/4 = 0.8272 (*in vacuo*). $n_D^{25} = 1.4367$.

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+2.93°}{1 \times 0.827} = +3.54°;\ [M]_D^{25} = +6.59° \text{ (homogeneous)}$$

$$\text{Maximum } [M]_D^{25} = +17.4° \text{ (homogeneous)}$$

3.040 mg. substance: 8.625 mg. CO_2 and 3.820 mg. H_2O

$C_{12}H_{26}O$.	Calculated.	C 77.3, H 14.1
186.2	Found.	" 77.4, " 14.1

3-Methyl-5-Iodoundecane—11 gm. of 3-methylundecanol-5, $[\alpha]_D^{25} = +3.54°$ (homogeneous), were placed in a bomb tube and cooled in a dry ice-acetone bath, and about 30 cc. of anhydrous hydrogen iodide were distilled into it. The tube was sealed and let stand at room temperature for 4 days. It was again cooled and opened. It was then allowed to come to room temperature overnight and then let stand for an additional 3 days in order to allow the excess hydrogen iodide to evaporate. The iodide was extracted with pentane, and the extracts were washed with dilute carbonate and water, and dried over calcium chloride and distilled. B.p. 88° at 1 mm. pressure. Yield 15 gm. *D* 25/4 = 1.1972 (*in vacuo*).

$$[\alpha]_D^{25} = \frac{+7.00°}{1 \times 1.197} = +5.85°;\ [M]_D^{25} = +17.3° \text{ (homogeneous)}$$

$$\text{Maximum } [M]_D^{25} = +45.7° \text{ (homogeneous)}$$

3.620 mg. substance: 6.470 mg. CO_2 and 2.760 mg. H_2O

$C_{12}H_{25}I$.	Calculated.	C 48.6, H 8.5
296.1	Found.	" 48.7, " 8.5

Methylethyl-n-Octylmethane—15 gm. of 3 methyl-5-iodoundecane, $[\alpha]_D^{25} = +5.85°$ (homogeneous), were reduced with Raney's

catalyst in methyl alcohol and sodium hydroxide solution. This was shaken in an atmosphere of hydrogen for 48 hours. The *hydrocarbon was extracted with pentane, and the extracts were* washed with water and concentrated calcium chloride solution and dried over metallic sodium. The product was then distilled. B.p. 94° at 15 mm. pressure. Yield 7 gm. *D* 25/4 = 0.7491 (*in vacuo*). $n_D^{25} = 1.4216$.

$$[\alpha]_D^{25} = \frac{+2.90°}{1 \times 0.749} = +3.87°; \; [M]_D^{25} = +6.59° \text{ (homogeneous)}$$

Maximum $[M]_D^{25} = +17.4°$ (homogeneous)

4.410 mg. substance: 13.690 mg. CO_2 and 6.100 mg. H_2O

$C_{12}H_{26}$.	Calculated.	C 84.6, H 15.4
170.2	Found.	" 84.7, " 15.5

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OPTICAL ROTATION OF CONFIGURATIONALLY RELATED ALDEHYDES

By P. A. LEVENE and ALEXANDRE ROTHEN

With the Assistance of Martin Kuna

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, August 8, 1935)

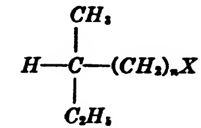
Two events were observed in the series of carboxylic acids of the general type

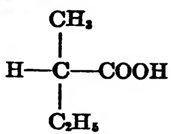
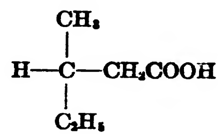
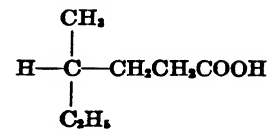
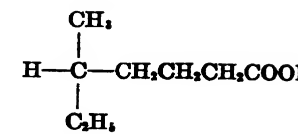
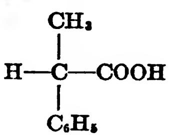
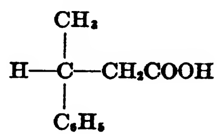
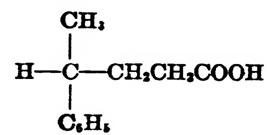
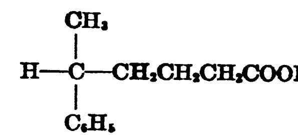
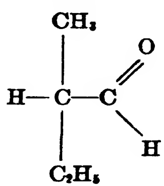
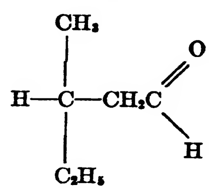
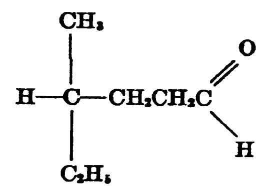
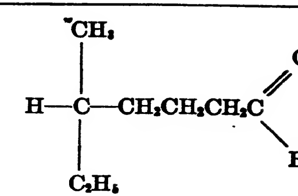
$$\begin{array}{c} \mathrm{CH_3} \\ | \\ \mathrm{H{-}C{-}(CH_2)_nCOOH} \\ | \\ \mathrm{R} \end{array}$$

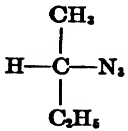
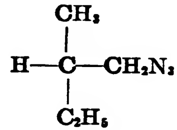
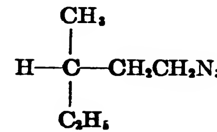
(R = normal alkyl or $(CH_2)_mC_6H_5$; m and n = 0 or an integer). First, the sign of the partial rotation of the COOH group in the purely aliphatic and probably also in the phenyl acids changed when n passed from 0 to 1, the sign remaining constant with further increase in the value of n. Second, there was observed a periodicity in the maximum values of the optical rotations in the visible spectrum with the consecutive increase in the values of n.

The nearest anisotropic absorption band of these substances was located comparatively far in the ultra-violet region. It was therefore of interest to study the effect of the distance from the asymmetric center of a chromophoric group with an absorption band in the nearer ultra-violet region. The aldehydes were chosen, inasmuch as the first absorption band of $-\mathrm{C}\left\langle\begin{matrix}\mathrm{O}\\ \mathrm{H}\end{matrix}\right.$ is in the neighborhood of λ 2900. It may be mentioned here that in the acids containing a phenyl group anisotropy could not be detected in the nearest absorption region of the phenyl group

Table I

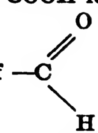
Maximum $[M]_D^{25}$ *of Configurationally Related Substances of the Type* 

 +18.0°*	 +10.4°†	 +13.6°†	 +11.1°†
 +111.6°	 −81.3°	 −39.3°	 −47.5°
 +20.3°‡	 −8.7°‡	 +12.0°	 +12.9° (Approximate)

		
⋝ −16.0°§	+10.9°§	+26.3°§

* Partial rotation of COOH dextro.

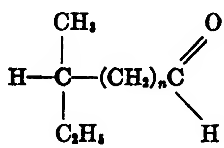
† Partial rotation of COOH levo.

‡ Partial rotation of  has the sign of the observed rotation.

§ The anisotropic band of N_3 is situated in the neighborhood of λ 1800 to λ 1900. The band at λ 2880 is not active.

(λ 2700 to 2500). The N_3 group in organic azides and the iodine atom in organic iodides have likewise absorption bands in the near ultra-violet region; N_3 about λ 2880, and iodine about λ 2600. The group of azides has, however, the disadvantage that the substances in which $n = 0$ and 1 are correlated only on theoretical considerations. In the case of iodides of the same general type, the substances in which $n = 0$ and 1 cannot be correlated either by direct chemical means or by any rigorous theoretical method.

Four aldehydes of the type

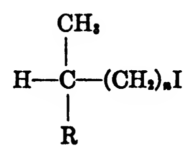

($n = 0$ to 3)

were prepared. In Table I their maximum rotations are compared with those of the carboxylic acids, azides, and iodides of the same general type.

From Table I it may be seen that in the aldehydes the partial rotation of the $-\mathrm{C}\Big\langle^{O}_{H}$ group (due to the band λ 2900) changes its sign when n passes from 0 to 1. The direction of this partial rotation remains constant in substances in which $n = 2$ and 3, since their dextrorotation is due to the high value of the partial contribution of the rest of the molecule, and not to the contribution of the first band of $-\mathrm{C}\Big\langle^{O}_{H}$ which is levorotatory. The fact, however, that the third member is dextrorotatory shows that there is an unmistakable periodicity in the rotatory values of the first three members. There is a probability that the true maximum rotation of the fourth member is smaller than that of the third, the reason being that the starting material for the synthesis of the aldehydes was the fermentation amyl alcohol. In this as well as in the bromide derived from it the true proportion of the optically active substances and of the isoamyl derivatives can easily be calculated. It is, however, not certain that the proportion remains the same in the aldehydes. It may be mentioned that the rotation of a syn-

thetic active amyl bromide in isoamyl bromide was found to differ but slightly from that of the homogeneous bromide.

The importance of the observations on the aldehydes lies in the fact that because of the proximity of the first absorption band in the ultra-violet region the analysis of their rotatory dispersion curves can be made with greater rigor than that of the carboxylic acids. The fact that the events in the aldehydes are similar to those in the acids adds credence to the assumption made in regard to the configurational relationships of the primary with the secondary azides and also suggests the possibility that the partial rotation of the iodine atom in substances of the type



in which $n = 0$, is of opposite sign from that of the iodine atom of substances in which n = an integer.

Synthesis of the Aldehydes—2-Methylbutanal-1 was prepared according to the procedure of Felix Ehrlich as described by Levene.[1]

The higher aldehydes were prepared by the method of Tschitschibabin.[2] However, in order to obtain satisfactory yields a special procedure was followed: The neutral freshly distilled orthoformic ester was brought to a boil in a three-neck flask provided with a mechanical stirrer, condenser, and dropping funnel. To the hot ester the Grignard reagent was added at a slow rate in order to maintain gentle refluxing. The reaction product was allowed to stand at room temperature overnight and the further treatment was the conventional one.

Table II gives an account of the intermediate steps leading to the higher aldehydes.

EXPERIMENTAL

Methylethylacetaldehyde (2-Methylbutanal-1)—The aldehyde was prepared from Kahlbaum's amyl alcohol ($\alpha = -1.1°$) according

[1] Levene, P. A., *J. Biol. Chem.*, **110**, 323 (1935).
[2] Tschitschibabin, A. E., *Ber. chem. Ges.*, **37**, 186 (1904).

Table II

$[M]_D^{25}$ *of Intermediates Leading to* $H-C(CH_3)(C_2H_5)-(CH_2)_nC(=O)H$

Unless otherwise indicated the rotations were taken in homogeneous state.

$H-C(CH_3)(C_2H_5)-$	$-CH_2OH$	$-C(=O)H$	$-CH_2Br$	$-CH_2C(=O)H$	$-CH_2CH_2Br$	$-CH_2CH_2C(=O)H$	$-CH_2CH_2CH_2Br$	$-CH_2CH_2CH_2C(=O)H$
$[M]_D^{25}$	−1.18	+9.54 Heptane	+2.24	−2.46 Heptane	+6.35	+1.97 Heptane	+4.35	+2.56 Heptane
$[M]_D^{25}$ maximum	−5.2	+20.3* (Approximate)	+7.9	−8.7 Heptane (approximate)	+38.8	+12.0 Heptane (approximate)	+21.9	+12.9 Heptane (approximate)

* Ehrlich, F., *Ber. chem. Ges.*, **40**, 2538 (1907).

to Ehrlich, as described by Levene.[1] B.p. 90–92°, p = atmospheric.

$$[\alpha]_D^{25} = \frac{+5.10° \times 100}{4 \times 11.51} = +11.1°; [M]_D^{25} = +9.54° \text{ (heptane)}$$

Maximum $[\alpha]_D^{20} = +23.56°$;* $[M]_D^{20} = +20.3°$ (homogeneous) (approximate)

4.870 mg. substance: 12.440 mg. CO_2 and 5.100 mg. H_2O

$C_6H_{10}O$.	Calculated.	C 69 70, H 11.71
86.1	Found.	" 69.65, " 11.71

In order to determine the rotation of active amyl bromide, in a solution of isoamyl bromide, 0.9890 gm. of active amyl bromide,

$$[\alpha]_D^{25} = \frac{-1.75°}{1 \times 1.21} = -1.44° \text{ (homogeneous)}$$

was made up to 2.5 cc. with isoamyl bromide,

$$[\alpha]_D^{25} = \frac{-0.53° \times 100}{1 \times 39.6} = -1.34°$$

The difference of the two rotations is within the limits of experimental error.

Methylethylpropionaldehyde (*3-Methylpentanal-1*)—34 gm. of active amyl bromide, $[M]_D^{25} = +2.24°$ (homogeneous), were dropped into 10 gm. of magnesium turnings in 85 cc. of dry ether. The reaction product was refluxed for 10 minutes and filtered through a Schott No. G-1 glass filter. The filtrate was slowly dropped into 100 gm. of boiling ethyl orthoformate with stirring. The solution was refluxed an additional hour and then let stand overnight. The next day it was poured into ice and ammonium chloride solution and extracted with ether. The extract was washed with ammonium chloride solution and the ether removed by distillation. The residue was hydrolyzed with 50 cc. of 5 N sulfuric acid for 45 minutes. The product of hydrolysis was poured onto ice and extracted with ether. The ether extract was shaken overnight with a saturated solution of sodium bisulfite. The crystalline bisulfite compound was filtered off, exhaustively washed with ether, and steam-distilled from 20 per cent potassium carbonate. The distillate was extracted with ether, and the extract was washed with concentrated calcium chloride solution

* Ehrlich, F., *Ber. chem. Ges.*, 40, 2538 (1907).

and dried over sodium sulfate. The aldehyde was distilled B.p. 122°, p = atmospheric. D 25/4 = 0.8010 (*in vacuo*); n_D^{25} = 1.4001.

The rotation was

$$[\alpha]_D^{25} = \frac{-1.32° \times 100}{4 \times 13.40} = -2.46°; [M]_D^{25} = -2.46° \text{ (heptane)}$$

$$\text{Maximum } [M]_D^{25} = -8.7° \text{ (heptane)}$$

4.120 mg. substance: 10.890 mg. CO_2 and 4.410 mg. H_2O

$C_6H_{12}O$.	Calculated.	C 71.93, H 12.09
100.1	Found.	" 72.08, " 11.97

Methylethylbutyraldehyde (4-Methylhexanal-1)—60 gm. of methylethylpropyl bromide, $[M]_D^{25}$ = +6.35° (homogeneous), were dropped into 12 gm. of magnesium in 150 cc. of dry ether. This bromide was derived from Kahlbaum's amyl alcohol through the usual intermediates whose rotations were as follows:

Amyl alcohol, $\alpha_D^{25} = -1.55° \rightarrow$ amyl bromide, $\alpha_D^{25} = +0.90° \rightarrow$ methylethylpropanol, $\alpha_D^{25} = +1.40° \rightarrow$ methylethylpropyl bromide, $\alpha_D^{25} = +4.50°$; $[M]_D^{25} = +6.35°$ (homogeneous).

The reaction product was heated 10 minutes, filtered through a No. G-1 funnel, then slowly dropped into 200 gm. of boiling ethyl orthoformate, refluxed for 1 hour, and let stand overnight. The aldehyde was isolated in the manner described for the methylethylpropionaldehyde. B.p. 144°, p = atmospheric. Yield 6.5 gm. D 25/4 = 0.8132 (*in vacuo*); n_D^{25} = 1.4081.

$$[\alpha]_D^{25} = \frac{+0.80° \times 100}{4 \times 11.59} = +1.73°; [M]_D^{25} = +1.97° \text{ (heptane)}$$

$$\text{Maximum } [M]_D^{25} = +12.0° \text{ (heptane)}$$

4.010 mg. substance: 10.800 mg. CO_2 and 4.430 mg. H_2O

$C_7H_{14}O$.	Calculated.	C 73.61, H 12.37
114.1	Found.	" 73.44, " 12.36

Methylethylvaleraldehyde (5-Methylheptanal-1)—79 gm. of methylethylbutyl bromide, α_D^{25} = +2.60° (homogeneous), were added to 20 gm. of magnesium turnings in 200 cc. of dry ether. This bromide was derived from the methylethylpropyl bromide described above. The rotations of the intermediates were as follows:

Methylethylpropyl bromide, $\alpha_D^{25} = +4.50° \rightarrow$ methylethylbutanol, $\alpha_D^{25} = +1.56° \rightarrow$ methylethylbutyl bromide, $\alpha_D^{25} = +2.60°$; $[M]_D^{25} = +4.35°$ (homogeneous).

After all the bromide was added, the solution was refluxed for 10 minutes and filtered through a Schott No. G-1 glass filter. The filtrate was then added dropwise to 225 gm. of boiling ethyl orthoformate with stirring. The solution was refluxed for 1 hour and then let stand overnight at room temperature. The aldehyde was isolated through its bisulfite compound, as described for the other aldehydes. B.p. 72°, $p = 25$ mm. Yield 18 gm. D 25/4 = 0.8164 (*in vacuo*); $n_D^{25} = 1.4144$.

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+1.58° \times 100}{10 \times 7.90} = +2.00°; [M]_D^{25} = +2.56°$$

$$\text{Maximum } [M]_D^{25} = +12.9° \text{ (heptane)}$$

3.885 mg. substance: 10.660 mg. CO_2 and 4.370 mg. H_2O

$C_8H_{16}O$.	Calculated.	C 74.92, H 12.59
128.1	Found.	" 74.77, " 12.58

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Vol. 4, No. 1, pp. 48–52]

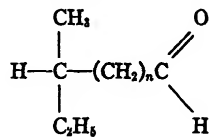
ROTATORY DISPERSION OF ALIPHATIC ALDEHYDES

BY P. A. LEVENE AND ALEXANDRE ROTHEN

(From the Laboratories of The Rockefeller Institute for Medical Research)

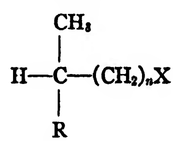
(Received for publication, October 11, 1935)

Rotatory dispersion curves of configurationally related aldehydes of the type



(n being equal to 0, 1, 2, or 3) have been determined in the visible and the ultraviolet regions of the spectrum. Their analysis has shown first, that the band of the aldehydic group at λ2950 is rotatory active and that the sign of its contribution in the first member ($n = 0$) is opposite to that of the higher members and second, that the magnitude of that contribution varies periodically with the number of carbon atoms located between the aldehydic group and the asymmetric carbon atom.

Previous observations in this laboratory[1] have established the fact that in substances of the general type



(R = an alkyl or aryl group, n = 0 or an integer, X = a chromophoric group with an absorption region situated in the near ultraviolet region of the spectrum) the change of the value of n from 0 to 1 brings about a change in the direction of rotation, this direction remaining constant for the substances with higher values of n. In the few cases[2] where no change of sign was observed, the analysis of the dispersion curve showed that the dispersion was anomalous, i.e., the sign of the rotation was opposed to that of the first contribution. These facts led to the

[1] P. A. Levene and R. E. Marker, J. Biol. Chem. **103**, 299 (1933).

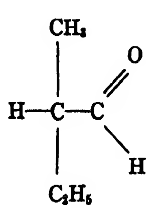
[2] P. A. Levene, A. Rothen and R. E. Marker, J. Chem. Phys. **1**, 662 (1933).

belief that in all other cases, the observed changes of rotation were due to changes in the first contribution. Further, there was observed a periodicity in the values of the rotations of the individual members with the progressive increase in the value of *n*.

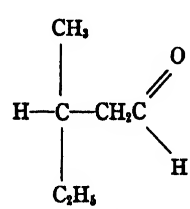
The group of substances thus far analyzed, however, was of a nature which did not permit direct measurement of the course of rotation within the region of the nearest absorption band. Hence the direction and value of its partial rotation were obtained by the analysis of the rotatory dispersion curve outside the band.

It was therefore desirable to analyze a group of substances of the same general type but with a chromophoric group which permitted the measurement of its rotation within the region of the absorption band. The group $-\mathrm{C}\Big\langle^{//}_{\mathrm{H}}$ held out the promise of presenting these advantages. The first absorption band of this group at 2950A is weak (E ≅ 20) well isolated without any overlapping, thus permitting calculation of its partial rotation from the experimental dispersion curve within the region of the absorption band.

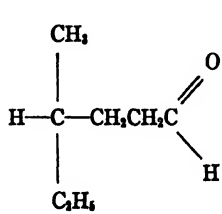
Hence, the following series of configurationally related aldehydes of the above general type were prepared:



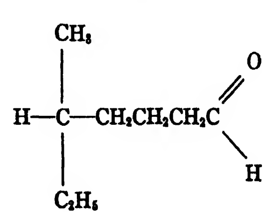
$[M]^{25}_{D}$ max. = +20.3°



$[M]^{25}_{D}$ max. = −8.7°



$[M]^{25}_{D}$ max. = +12.0°



$[M]^{25}_{D}$ max. = +12.8°

The mode of preparation of these substances has been described elsewhere.[3] The present communication deals with the analysis of the rotatory dispersion curves of the above four aldehydes. The results of the observations are summarized in Fig. 3. The ordinate represents the absolute magnitude of the partial rotation (which is proportional to the circular dichroism) of the band λ2950, the abscissa the number of carbon atoms between the asymmetric center and the aldehydic group (the numerical value of n).

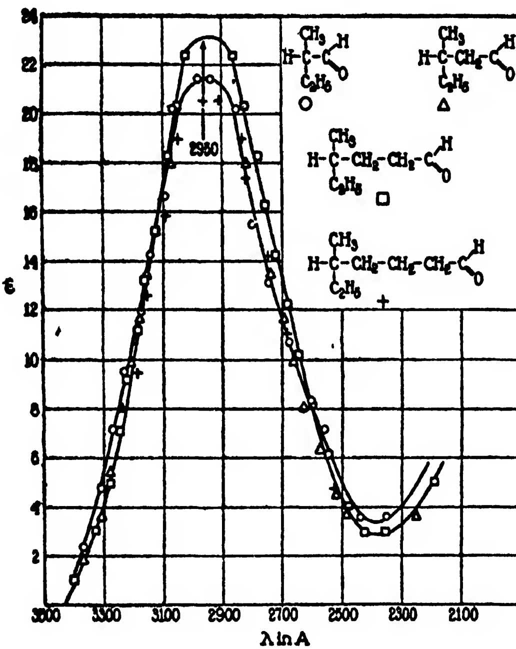


FIG. 1

It is evident that there is a periodicity in the value of the rotatory component of the band λ2950. This partial rotation is comparatively small in the third and fourth members (n = 2 and 3). However, its magnitude in the fourth member is twice that of the third. Thus it is clear that the periodic variations of rotation with the increase of the numerical value of n, observed in the visible part of the spectrum are due to periodic variations in the partial contribution of the band

[3] P. A. Levene and A. Rothen, J. Biol. Chem. **111**, 739 (1935).

λ2950. These observations are significant for they add weight to the conclusions reached earlier regarding substances whose nearest absorption bands were not accessible to direct analyses.[4]

The physical basis of the observed changes in rotation still remains a matter of speculation. Among all the theories concerning optical activity, none is able to predict, first, that a change of sign occurs in the function $(\varphi)_\lambda{}^* = f(n)$ when n is changed from 0 to 1; second, that for $n > 1$ this function keeps the same sign but varies periodically with the successive values of n in magnitude only. It is possible to deduce from the mechanical model used by W. Kuhn[5] that a change of sign should occur when the distance between the two coupled oscillators increases, but the wave-length should be of the order of magnitude of interatomic distances, which is, of course, excluded in the case here considered. The more recent theory of Born[6] shows that one could expect a short periodicity in the function $(\varphi)_\lambda = f(n)$ but no change of sign can be predicted.

In the absence of any other explanation for the observed facts, we venture to suggest one which is in harmony with the deduction from Kuhn's mechanical model and with the theoretical consideration of Born[6] and others. The facts to be remembered are: First, that the configuration remaining constant, it is only the change of n from 0 to 1 which brings about inversion of sign of a given $(\varphi)_\lambda$, the sign remaining the same in all the other members of a homologous series. Second, there is no change in direction of rotation and no periodicity if $X = CH_3$ so long as the length of the chain $(CH_2)_nCH_3$ does not exceed that of R. Thus it is evident that the polar nature of X is a requisite for the phenomenon. On the other hand, it is known that the polar

[4] In an interesting and recent article, H. Hudson, M. L. Wolfrom and T. M. Lowry, J. Chem. Soc. 1179 (1933), studied the rotatory dispersion of aldehydic sugars. They report a strong partial rotation of the band λ2900 which, in one case, is practically the only rotatory component of the rotation observed. But the presence, in those compounds, of three or four asymmetric carbon atoms renders interpretation more difficult.

* The symbol $(\varphi)_\lambda$ will be used to denote the partial rotatory contribution of the band λ due to the group X.

[5] W. Kuhn and K. Freudenberg, "Drehung der Polarisationsebene des Lichtes," *Hand- und Jahrbuch der chemischen Physik* (Leipzig, 1932), p. 47.

[6] M. Born, Proc. Roy. Soc. **A869**, 84 (1935).

groups induce alternating polarities in the consecutive carbon atoms of the chain, which naturally is extended to the asymmetric carbon atom. Hence it is reasonable to assume that the dissymmetry of the asymmetric carbon atom will alter with the change of the electric charge of the neighboring carbon atom.

TABLE I

Rotatory Dispersion of Dextro-2-Methylbutanal-1 in Heptane

Concentration: 1.337M. Visible region: l = 40 cm. U.V.: l = 5 cm from 3640 to 3460A, 1 cm for 3420A, 0.1 cm for λ <3420.

λ(A)	α^{25}	$[M]^{25}_{max.}$	$[M]^{25}_{max.} = \frac{5.104}{\lambda^2 - 0.0932}$
5875.6	+5.140	+20.28	+20.25
5780.1	+5.369	+21.19	+21.19
5460.7	+6.315	+24.92	+24.92
4358.3	+13.37	+52.76	+52.76
4046.6	+18.30	+72.21	+72.36
3640	+3.98	+125	+130
3588	+4.48	+141	+144
3540	+4.99	+157	+159
3500	+5.49	+173	+174
3460	+6.00	+189	+188
3420	+1.35	+215	+215
3320–3070	+0.20	+325	—
2980	0.00	0	—
2930	−0.10	−160	—
2770	−0.35	−450	—
≌2350	−0.15	−240	—

Analysis of the Absorption Band

The absorption curves of the aldehydes may be seen in Fig. 1. It is apparent that the position of the band is identical in all four compounds, the intensity being of the same order of magnitude. The absorption curves can be expressed by an exponential equation based on a Maxwellian distribution.

Analysis of Rotatory Dispersion Curve

The measurements of the rotatory dispersions may be found in Tables I, II, III, IV, and on Fig. 2. The values found in the fourth

column of the tables have been calculated, using in one case a one-Drude term, and in the others a two-Drude term formula. The constants of the formulas have been so chosen as to reproduce as well as

TABLE II

Rotatory Dispersion of Levo-3-Methylpentanal-1 in Heptane

Constants of sample: δ_4^{25} 0.8010, n_D^{25} 1.3989. Concentration 1.338M. Visible region: l = 40 cm. U.V.: l = 10 cm from 3970 to 3475A 1 cm for a, 0.5 cm for b, 0.1 cm for c

λ(A)	α^{25}	$[M]^{25}_{max.}$	$[M]^{25}_{max.} = -\frac{7.569}{\lambda^2-0.093} + \frac{6.693}{\lambda^2-0.032}$
5875.6	−1.320	−8.67₉	−8.64
5780.1	−1.409	−9.26₄	−9.24
5460.7	−1.786₅	−11.74₆	−11.75
4358.3	−5.43	−35.7	−35.7
4046.6	−8.54	−56 1₆	−56.1
3885	−2.75	−72.3	−74.3
3850	−3 00	−78.9	−79.5
3710	−4.00	−105	−106
3660	−4.50	−118	−119
3630	−5.00	−131	−128
3590	−5.50	−144.₅	−142
3555	−6.00	−158	−156
3535	−6.50	−171	−165
3510	−7.00	−184	−177
3492	−7.50	−197	−188
3475	−8.00	−210	−198
3430	−1.11 (a)	−292	
3420	−1.17 (a)	−323	
3395	−1.25 (a)	−329	
3360	−0.76 (b)	−400	
3320	−0.70 (b)	−460	
3200	−0.22 (c)	−580	
3000	−0.00 (c)	0.0	
2730	+0.43 (c)	+1100	
2660	+0.33 (c)	+870	
2500	+1.26 (b)	+670	
2400	+2.46 (a)	+650	

possible the experimental data in the visible and very near ultraviolet regions. The agreement between experimental and calculated values is quite satisfactory especially if one considers that in the case of 3-

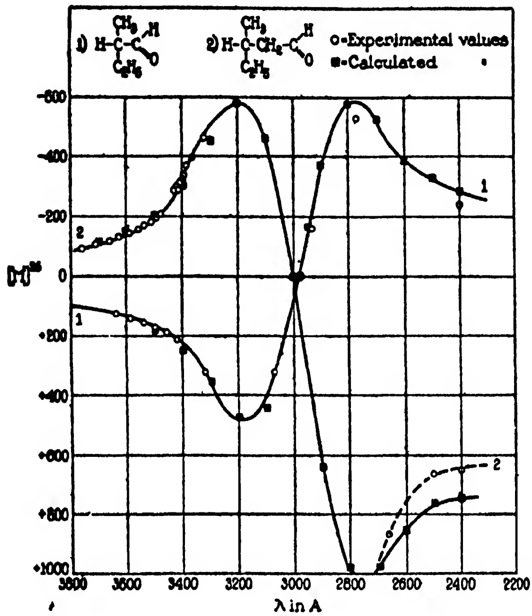

o-Experimental values
■-Calculated
-600
-400
-200
0
+200
+400
+600
+800
+1000
3800
3600
3400
3200
3000
2800
2600
2400
2200
λ in A

FIG. 2

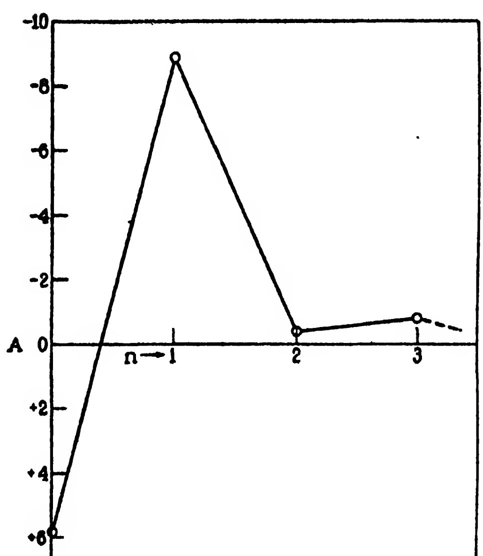

-10
-8
-6
-4
-2
A 0
+2
+4
+6
n → 1
2
3

FIG. 3

methylpentanal-1 the two terms of opposite sign are of the same order of magnitude. The dispersion constants of the first terms come very

TABLE III

Rotatory Dispersion of Dextro-4-Methylhexanal-1 in Heptane

Constants of sample: δ_4^{25} 0.8132, n_D^{25} 1.4081. Concentration 1.016M. Visible region: l = 40 cm. U.V.: l = 10 cm for 3490A and 0.5 cm for 2350A.

λ(A)	α^{25}	$[M]^{25}_{max.}$	$[M]^{25}_{max.} = -\frac{0.371}{\lambda^2 - 0.087} + \frac{4.114}{\lambda^2 - 0.036}$
5875.6	+0.800	+12.01	+11.87
5780.1	+0.819	+12.30	+12.30
5460.7	+0.930	+13.96	+13.93
4358.3	$+1.54_0$	$+23.1_2$	+23.12
4046.6	+1.82	+27.3	+27.37
3490	+0.60	+36	+37
2350	+0.20	+240	+228

TABLE IV

Rotatory Dispersion of Dextro-5-Methylheptanal-1 in Heptane

Constants of sample: δ_4^{25} 0.8164, n_4^{25} 1.4144. Concentration 0.616M. Visible region: l = 100 cm. U.V.: l = 20 for 3770 and 3580A, 5 cm for 3450A, 1 cm for 2460A.

λ(Å)	α^{25}	$[M]^{25}_{max.}$	$[M]^{25}_{max.} = -\frac{0.8158}{\lambda^2 - 0.087} + \frac{4.9102}{\lambda^2 - 0.036}$
5875.6	+1.596	+12.79	+12.72
5780.1	+1.643	+13.17	+13.17
5460.7	+1.856	+14.88	+14.86
4358.3	+2.99	$+23.9_7$	+23.97
4046.6	+3.47	$+27.8_2$	+27.81
3770	+0.80	+32	+31.5
3580	+0.90	+36	+33.5
3450	+0.20	+32	+33.6
2460	+0.30	+240	+230

close to the position of the absorption band, with a slight displacement towards the red. The dispersion curves of the first two compounds

(n = 0 and 1) are reproduced in Fig. 2. It was impossible to obtain a two-term formula representing accurately the experimental data over the whole wave-length range covered. The smallness of the angles observed as well as a gradual decrease in the rotations due to decomposition prevented any accurate determinations in the immediate neighborhood of the band. The calculated values have been obtained from a two-term formula, the first term being a Kuhn-Braun[7]-Lowry-Hudson[8] term. The general formula used was

$$[M]^{25}_{\max.} = \frac{\phi\lambda_\phi}{m\lambda}\left[e^{-[(\lambda-\lambda_0)/\theta]^2}\int_0^{(\lambda-\lambda_0)/\theta} e^{x^2}dx + \frac{\theta}{2(\lambda+\lambda_0)}\right] + \frac{B}{\lambda^2-\lambda_1^2},$$

where: $\phi = +500°$, $m = 0.560$, $\lambda_\phi = 0.3166$, $\lambda_0 = 0.2980$, $\theta = 0.0207$, $B = -0.798$, $\lambda_1^2 = 0.036$ for levo 2-methylbutanal-1 and $\phi = -700°$, $m = 0.560$, $\lambda_\phi = 0.3180$, $\lambda_0 = 0.2980$, $\theta = 0.0223$, $B = +8.07$, $\lambda_1^2 = 0.036$ for dextro-3-methylpentanal-1. (To comply with the usual convention adopted in polarimetry, wave-lengths are expressed in microns.) As known,[7] the first term of this formula reduces to a simple Drude term $A/(\lambda^2 - \lambda_0^2)$ for values of $\lambda > 4\theta+\lambda_0$ where $A = \phi\lambda_\phi\theta/m$.

The following facts should be brought out: First, the center of the active part of the band is slightly displaced towards the red as compared to the absorption band; second, the active band does not spread over the entire width of the absorption band: The constants θ determined from absorption measurements are very much larger than θ determined to fit the dispersion formulas; third, the high value of the dispersion constants of the second terms for all compounds indicate that the second contribution is also located for the most part in the aldehydic group.

Experiments were attempted to determine directly the dichroism in the band λ2950. As expected, it could be detected only in the first two compounds. The measurements remained of a qualitative nature because of the small angles, but were, however, of the right order of magnitude.

[7] W. Kuhn and E. Braun, Zeits f. physik. Chemie **B8**, 281 (1930).

[8] T. M. Lowry and H. Hudson, Phil. Trans. Roy. Soc. London **A232**, 117 (1933).

EXPERIMENTAL

The instruments used have been previously described[9] and the same precision is claimed. The method followed to determine the circular dichroism was that described by W. Kuhn.[10]

The compounds used were not resolved to the maximum. All observed α values as given in the tables were multiplied by an appropriate coefficient to bring them to the maximum. The decrease of rotation due to decomposition was taken into account when the series of measurements extended over a long period of time.

[9] P. A. Levene and A. Rothen, J. Chem. Phys. **2**, 681 (1934).

[10] W. Kuhn and E. Braun, Zeits. f. physik. Chemie **B8**, 445 (1930).

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THE OXIDATION OF *dl*-α-HYDROXYSTEARIC ACID AND ITS SIGNIFICANCE AS REGARDS THE STRUCTURE OF CEREBRONIC ACID

A Reply to the Paper of Klenk and Ditt*

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(Received for publication, May 31, 1935)

After reading the preceding article by Klenk and Ditt we realize that the conclusion of the work of Levene and Yang was worded in a way permitting misunderstanding. It should have been stated, as in the article by Levene and West,[1] that on oxidation of α-hydroxystearic acid as well as of a synthetic α-hydroxytetracosanic acid, under our conditions of oxidation and working on small quantities of material only, the next lower acid was obtained in a state approaching purity in a yield of over 80 per cent of the total oxidation products. That a small proportion of lower acids was present in the oxidation products is evident from the fact that the average molecular weight of the smaller fraction was given as 261, whereas the larger fraction (80 per cent of the total) had an average molecular weight of 269 (theory for $C_{17}H_{34}O_2$, 270).

We wish, however, to emphasize that the work on oxidation of α-hydroxystearic acid was undertaken by us as a control for the work on cerebronic acid. In our hands the two acids did not behave identically. An impartial reader will have to conclude that also in the hands of Klenk and his coworkers the acids behaved differently. The differences have been pointed out in previous articles and need not be repeated.

* Klenk, E., and Ditt, F., *J. Biol. Chem.*, **111**, 749 (1935).

[1] Levene, P. A., and West, C. J., *J. Biol. Chem.*, **16**, 475 (1913–14).

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ON PROTEOLYTIC ENZYMES

VIII. THE PROTEOLYTIC SYSTEMS OF PAPAIN

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(Received for publication, July 27, 1935)

The favorable effect of HCN on papain, first noted by Vines (1), was established by Mendel and Blood (2) and Frankel (3) as an activation of the enzyme. Willstätter and Grassmann (4) in a quantitative investigation determined the proteolytic behavior of the natural and HCN-activated enzyme. Natural papain split proteins and the activated form split both proteins and peptones. Both effects were attributed to the same individual enzyme, whose range of specificity was extended by activation. Grassmann, Ambros, Waldschmidt-Leitz, Maschmann, and Bersin have studied this activation. Bersin (5) has concluded that the activation process consists of a transformation of disulfide into sulfhydryl (thiol) groups. He regards the active enzyme as the thiol and the inactive as the disulfide form, and takes the number of thiol groups to be indicative of the activity of a given enzyme preparation.

There has recently been found in this laboratory (6, 7) a series of simple synthetic substrates for HCN-activated papain—acylated peptides such as carbobenzoxydiglycylglycine and even hippurylamide, which are readily hydrolyzed by papain-HCN. The problem was presented whether that enzyme system of papain which splits these synthetic substrates in the presence of HCN is identical with the system which hydrolyzes proteins. Therefore, with the help of the synthetic substrates, we have begun an investigation of the enzymic homogeneity and activation process of papain.

There was used in this research a crude papain obtained by evaporating *in vacuo* the juice of the *Carica papaya* fruit. Although this preparation was already effective in splitting gelatin, it was

found to be inactive upon such synthetic substrates as hippurylamide and carbobenzoxydiglycylglycine (Table I). The enzyme system of papain which attacks gelatin is therefore without effect on hippurylamide and carbobenzoxy tripeptides. If the partial activity of natural papain is due to an accompanying activator, this activator, whatever it is, does not or cannot activate the polypeptidase system we are studying.

In order to examine the effect of different activators, we have used hydrogen cyanide, thioglucose, and hydrogen sulfide with

TABLE I

Activation of Natural Papain

The increase is measured in ml. of 0.01 N KOH per 0.2 ml. of solution.

Activator	Substrate	Hydrolysis			
		2 hrs.	24 hrs.	50 hrs.	100 hrs.
	Gelatin	0.36	0.85	1.17	
		0.30	0.45	0.47	
	Hippurylamide	0.03*	0.05	0.02	0.04
		0.01	0.04	0.03	0.03
	Carbobenzoxytriglycine	0.01*	0.02	0.00	0.05
HCN	Gelatin	0.90	1.68	1.89	
		0.61	1.41		
"	Hippurylamide	0.16	1.05	1.02	
		0.21	1.06	1.08	
Thioglucose	Gelatin	0.64	1.38	1.33	1.50
"	Hippurylamide	0.05	0.70		1.03
H_2S	Gelatin	0.44	1.12	1.22	1.23
"	Hippurylamide	0.05	0.47	0.68	0.71

* 1.00 ml. represents 100 per cent splitting for all synthetic substrates.

crude papain. The effect of all these activators was the same (Table I); not only was the hydrolysis of gelatin increased, but a typical synthetic substrate like hippurylamide was readily split.[1] These activators therefore effect an extension of the specificity range of the papain aggregate analogous to that observed by Willstätter and Grassmann.

Experiments were then performed in which papain was first completely inactivated by oxidation and then treated with the

[1] HCN-activated papain did not split the typical dipeptidase, aminopeptidase, and carboxypeptidase substrates.

usual activators. The inactive papain was prepared by treating a solution of the enzyme with hydrogen peroxide and isolating the product by the addition of alcohol (Bersin and Logemann (8)). It was then completely inactive upon gelatin, as well as upon the synthetic acylated substrates (Table II). After reactivation with HCN, thioglucose, or thioglycolic acid gelatin was readily split, but neither hippurylamide nor carbobenzoxydiglycylglycine was attacked. Completely analogous were experiments in which iodine-oxidized papain was reactivated with hydrogen cyanide. Synthetic substrates were not hydrolyzed (Table III). The iodine-oxidized enzyme was not isolated from the solution.

As a result of these experiments one can differentiate between four stages of activity for papain as follows: (1) natural enzyme, effective on gelatin, without effect on synthetic substrates (or peptones), but capable of being activated for the latter (potential activity for synthetic substrates); (2) activated natural enzyme, effective both on gelatin and synthetic substrates (also peptones); (3) oxidized enzyme (H_2O_2 or I_2), no activity upon either of the above substrates, but with potential activity for gelatin; (4) enzyme activated after oxidation, effective on gelatin but ineffective and incapable of being activated for synthetic substrates.

The activation processes of papain for gelatin and for the synthetic substrates are independent of each other. Therefore, there must be in natural papain two different proteolytic enzymes, a proteinase and a polypeptidase, which differ not only in their substrates but also in their behavior on oxidation. In contrast to the reversible activity of the proteinase, that of the polypeptidase is, after oxidation, not reversible. It cannot be explained by a simple disulfide-thiol system. In case there is such a system in the polypeptidase, it must be combined with other essential groups.

As an approach to the question whether there are carbonyl groups in the papain polypeptidase, its behavior with phenylhydrazine was studied. Phenylhydrazine was added to the HCN-activated papain solution before beginning a hydrolysis (Table IV). Under these conditions there was no splitting of hippurylamide, although gelatin was hydrolyzed at a rate only slightly less than that without the hydrazine. It is remarkable what an extremely small amount of phenylhydrazine (0.0006 mM per ml. of test

solution) is capable of preventing the splitting of the synthetic substrates.

TABLE II

Activation of Papain after Oxidation with Hydrogen Peroxide

The increase is measured in ml. of 0.01 N KOH per 0.2 ml. of solution.

Activator	Substrate	Hydrolysis			
		2 hrs.	24 hrs.	50 hrs.	100 hrs.
	Gelatin	−0.02	0.06	0.03	
	Carbobenzoxytriglycine	0.01	0.01	0.02	0.06
HCN	Gelatin	0.02	0.58		1.60
			0.97	1.47	1.64
"	Hippurylamide	0.01	0.05	0.00	0.02
"	Carbobenzoxytriglycine	−0.05	−0.06	−0.06	−0.08
		0.00	−0.04	−0.08	−0.13
Thioglucose	Gelatin	0.39	1.36	1.47	1.40
		0.22	0.91		1.31
"	Carbobenzoxytriglycine	0.02	0.03	0.04	0.02
		0.00	0.00	0.01	−0.02
Thioglycolic acid	Gelatin	0.45	0.76	0.75	

TABLE III

Activation of Papain after Oxidation with Iodine

The increase is measured in ml. of 0.01 N KOH per 0.2 ml. of solution.

Activator	Substrate	Hydrolysis		
		2 hrs.	24 hrs.	50 hrs.
	Gelatin	0.02	0.29	0.34 (72 hrs.)
		0.05	0.27	0.44
	Carbobenzoxytriglycine	0.00	0.03	0.06 (72 ")
	Hippurylamide	0.01	−0.02	0.00
HCN	Gelatin		1.40	1.73 (67 ")
		0.67	1.26	1.35
"	Carbobenzoxytriglycine	0.04	−0.02	0.08 (67 ")
"	Hippurylamide	0.02	−0.02	0.00
				0.12

In its effect on gelatin and synthetic substrates the phenylhydrazine-treated papain is comparable to stage (4) mentioned above. It differs from stage (4) in that the latter is obtained by

destroying a polypeptidase of *potential* activity, whereas it is formed by altering an already *active* polypeptidase.

It is also possible to differentiate between the proteinase and polypeptidase by adding iodine to a HCN-activated papain

TABLE IV

Effect of Phenylhydrazine on HCN-Papain

The increase is measured in ml. of 0.01 N KOH per 0.2 ml. of solution.

Reagent, mM per ml. test solution		Substrate	Hydrolysis			
HCN	C_6H_5NH-NH_2		2 hrs.	4.5 hrs.	24 hrs.	48 hrs.
0.018		Gelatin	0.90		1.68	1.89
0.018		Hippurylamide	0.21		1.06	1.08
0.018	0.0025	Gelatin	0.52		1.35	1.56
0.018	0.0025	Hippurylamide	0.00		−0.02	−0.01
0.018	0.005	Gelatin		0.75	1.31	1.54
0.018	0.005	Hippurylamide		0.00	−0.03	−0.08
0.018	0.005	Carbobenzoxytriglycine	−0.01		−0.08	−0.08
0.018	0.025	Gelatin		0.56	1.11	1.34
0.018	0.025	Hippurylamide		0.03	−0.02	0.01
0.044		Gelatin	0.95		1.65	1.83
0.044		Hippurylamide	0.20		0.94	0.96
0.044	0.0006	"	0.04		0.00	−0.04
0.044	0.005	Gelatin	0.57		1.35	1.47
0.044	0.005	Hippurylamide	−0.02		0.04	0.03

TABLE V

Iodine Oxidation of HCN-Activated Papain

The increase is measured in ml. of 0.01 N KOH per 0.2 ml. of solution.

Reagent, mM per ml. test solution		Substrate	Hydrolysis	
HCN	Iodine		18 hrs.	48 hrs.
0.018	0.0005	Gelatin	1.53	1.92
0.018	0.0005	Hippurylamide	−0.02	−0.01
0.018	0.008	Gelatin	0.03	0.10
0.018	0.008	Hippurylamide	0.04	0.00

solution. With an excessive amount of iodine both the proteinase and polypeptidase components are destroyed. However, when only a small amount of iodine (0.0005 mM per ml. of test solution) is used, then only the polypeptidase component is inhibited (Table V).

The essential groups of the polypeptidase are therefore much more sensitive toward iodine than those of the proteinase.

The effect of iodine and hydrogen peroxide indicates that there is an easily oxidized group in polypeptidase. The inhibition by phenylhydrazine, considered with this, suggests an aldehyde carbonyl. The oxidation of aldehyde groups by iodine or hydrogen peroxide is well established in alkaline medium. Whether it would occur in our case in slightly acid solution is uncertain. So we wish to regard the presence of an aldehyde group merely as a working hypothesis until further investigation.

The new enzyme which splits synthetic substrates has been called a polypeptidase, because it requires at least two peptide linkages within its substrates (6, 7). The relation of this enzyme to the peptone-splitting enzyme of Willstätter and Grassmann is now being studied. There is no evidence whether the essential groups of the proteinase and polypeptidase are contained in the same molecule or whether the two enzymes are two separate molecular individuals. This question, too, will be investigated.

EXPERIMENTAL

I. General Procedure for Enzyme Test Solutions

The test solution was prepared so that it contained for each ml. 40 mg. of water-free gelatin or 0.05 mM of synthetic substrate, 0.10 ml. of disodium citrate buffer, pH 5.0, and 0.2 ml. of enzyme solution, described below. (Carbobenzoxytriglycine was dissolved with an equimolecular amount of 1 N NH_4OH.) 0.2 ml. samples of this solution were titrated with 90 per cent alcoholic 0.01 N KOH with thymolphthalein. During an experiment the solutions were kept at 40°, and a pH of 5 was maintained.

II. Enzyme Preparations

Natural Papain—225 mg. of powdered papain were shaken 1 hour with 15 ml. of water, filtered, 25 ml. of disodium citrate buffer, pH 5.0, added, and the solution made to 50 ml. with water.

HCN-Activated Papain—To a 15 ml. solution from 225 mg. of papain were added 25 ml. of citrate buffer and 10 ml. of 1.2 per cent HCN; the solution was kept for 2 hours at 40° before use.

Thioglucose-Activated Papain—To 10 ml. of the natural papain

solution above were added 50 mg. of sodium thioglucose; the solution was kept for 2 hours at 40° before use.

H_2S-Activated Papain—To 10 ml. of the natural papain solution were added 55 mg. of $Na_2S \cdot 9H_2O$; the solution was kept for 2 hours at 40° before use.

H_2O_2-Oxidized Papain—To 300 ml. of solution from 10 gm. of papain were added 6.6 ml. of 30 per cent H_2O_2. After 20 minutes at 30° it was cooled, 1400 ml. of alcohol were added, and the product centrifuged off and dried over calcium chloride; 4.9 gm. were obtained.

112 mg. of the above oxidized papain were dissolved in 12.5 ml. of water and citrate buffer was added to 25 ml.

H_2O_2-Oxidized Papain with HCN—To an 8 ml. solution of 112 mg. of oxidized papain were added 12 ml. of citrate buffer and 5 ml. of 1.2 per cent HCN; this was kept for 4 hours at 40° before use.

H_2O_2-Oxidized Papain with Thioglucose—To a 4 ml. solution of 45 mg. of oxidized papain were added 2.5 ml. of citrate buffer and 40 mg. of sodium thioglucose; this was made to 10 cc. and stood 1 hour at 40° before use.

H_2O_2-Oxidized Papain with Thioglycolic Acid—0.005 mM of thioglycolic acid per ml. of enzyme test solution was employed.

Iodine-Oxidized Papain—To a 15 ml. solution from 225 mg. of papain were added 10 ml. of citrate buffer and 4.2 ml. of 0.05 N iodine in aqueous KI. This was made to 50 ml. with citrate buffer and allowed to stand 2 hours at room temperature before use.

Iodine-Oxidized Papain with HCN—To 20 ml. of the above solution were added 5 ml. of 1.2 per cent HCN; this stood 2 hours at 40°. Instead of 0.2 ml. of enzyme solution per ml. of test solution, 0.25 ml. were used.

III. Phenylhydrazine Experiments

HCN-activated papain was prepared as in Section II. 1.2 per cent HCN was used in the experiments in which the concentration of HCN was 0.018 mM per ml. of test solution; 3 per cent HCN in those in which the concentration was 0.044 mM. The required amount of phenylhydrazine was added to the HCN-papain and the solution allowed to stand 1 hour at room tempera-

ture before use. The test solutions were treated in the customary manner (Section I).

IV. Iodine Oxidation of HCN-Activated Papain

The HCN-activated papain described in Section II was used. In the first set of two experiments (0.0005 mM of iodine per ml.), 0.5 ml. of 0.05 N iodine was added to 5 ml. of cooled HCN-papain solution; this was allowed to stand 1 hour at room temperature, and then used as is customary for the test solution (Section I).

In the second set of experiments, 80 ml. of 0.05 N iodine were added to 50 ml. of HCN-papain solution, and after an hour used in the hydrolysis test. 0.52 ml. of this solution was contained in each ml. of test solution.

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SPALTUNG VON CLUPEAN DURCH VERSCHIEDENE TRYPSINPRÄPARATE

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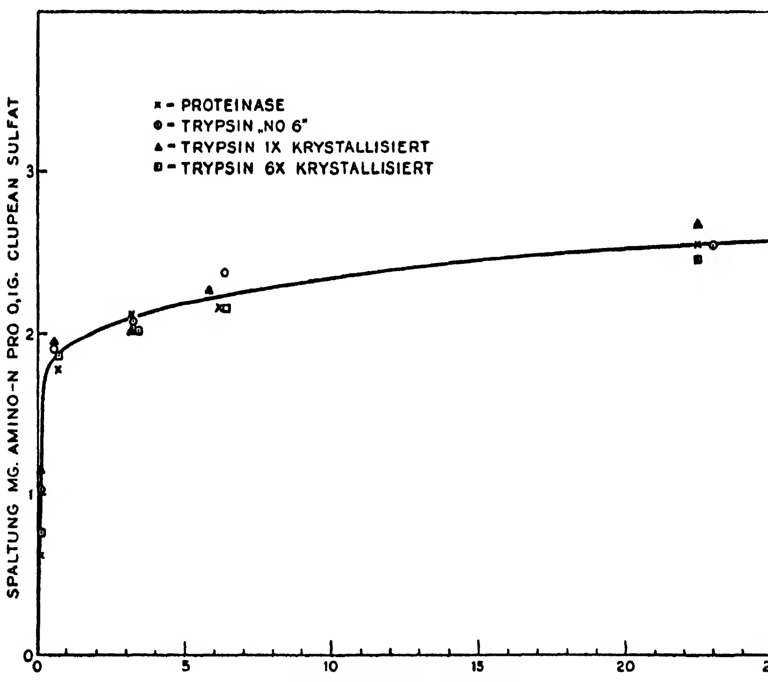
(Der Schriftleitung zugegangen am 11. Juni 1935)

Waldschmidt-Leitz und Akabori[1]) haben vor kurzem den Wirkungsbereich eines nach Adsorptionsmethoden gereinigten Proteinasepräparates aus Schweinepankreas mit dem eines nach Northrop und Kunitz[2]) aus Rinderpankreas dargestellten krystallisierten Trypsinpräparates verglichen. Eines der hierzu verwendeten Substrate war das von Waldschmidt-Leitz, Ziegler, Schäffner und Weil[3]) beschriebene Clupean, das Produkt der Einwirkung von Protaminase[3]) auf Clupein. Waldschmidt-Leitz und Akabori haben mit diesem Substrat festgestellt, daß der Spaltungsbereich des krystallisierten Trypsinpräparates den einer durch Aluminiumhydroxyd-Adsorption gereinigten Proteinase wesentlich übertraf, und haben daraus folgenden Schluß gezogen: „Der . . . Befund des wesentlich weiteren Wirkungsbereiches gegenüber Clupean für das Northropsche Trypsin mahnt zur Vorsicht; er scheint noch gegen die enzymatische Einheitlichkeit dieses Präparates zu sprechen."

Das Verfahren zur Darstellung und Reinigung krystallisierten Trypsins ist seit der Gewinnung des in der Arbeit von Waldschmidt-Leitz und Akabori untersuchten Präparates verbessert worden[4]) und wir haben daher den Vergleich der Spaltungsbereiche an Clupean nochmals wiederholt, da wir meinten, daß das seinerzeit von diesen Autoren untersuchte Trypsin vielleicht ein verunreinigendes Enzym enthalten haben könnte, und annahmen, daß die nach dem neueren Verfahren gewonnenen Präparate sich dem Zustand enzymatischer Einheitlichkeit, dessen Annahme durch das Studium anderer Eigenschaften des krystallisierten Trypsins nahegelegt worden war, mehr annähern würden.

*) Fellow der Rockefeller Foundation.

Das bei diesen Versuchen verwendete Clupeanpräparat, sowie eine Glycerinlösung von gereinigter[1]) Proteinase wurden uns von Herrn Prof. E. Waldschmidt-Leitz zur Verfügung gestellt, wofür wir ihm herzlichst danken. *Außer dieser Proteinaselösung (a) wurden untersucht: b) ein Trypsinpräparat, dargestellt nach*[2]), also in derselben Weise, wie das von Waldschmidt-Leitz und Akabori untersuchte krystallisierte Trypsin, und etwa gleichzeitig mit diesem; c) ein nach dem neueren Verfahren von Kunitz und Northrop[4]) dargestelltes, 1 mal krystallisiertes Präparat; d) dasselbe Präparat, 6 mal umkrystallisiert.



Wir hofften bei Verwendung derart verschieden weit gereinigter Präparate Gelegenheit zu haben, das allmähliche Verschwinden der vermuteten proteolytisch aktiven Verunreinigung zu beobachten. Wie das in der Figur zusammengestellte Ergebnis unserer Versuche zeigt, trat ein solcher Effekt jedoch nicht auf. Alle vier Enzympräparate ergaben denselben Spaltungsbereich; sein absoluter Betrag

entspricht fast genau dem von Waldschmidt-Leitz und Akabori für krystallisiertes Trypsin gefundenen; die Koordinaten der Figur sind den seinerzeit von diesen Autoren verwendeten angepaßt worden, um *einen direkten Vergleich mit*[1]) *Fig.* 4 *zu ermöglichen.* Den abweichenden Befund einer geringeren Spaltung durch Proteinase vermochten wir also an dem uns von Herrn Prof. Waldschmidt-Leitz übersendeten Präparat nicht zu bestätigen. Dies stimmt überein mit einem unveröffentlichten Befund von Dr. L. Weil, für dessen freundliche Privatmitteilung wir ebenfalls bestens danken, welchem zufolge ein anderes Proteinasepräparat gegenüber Clupean einen höheren Spaltungsbereich zeigte als den von Waldschmidt-Leitz und Akabori gefundenen.

Die von Waldschmidt-Leitz und Akabori untersuchte Proteinase enthielt, wie sie durch Spaltungsversuche an Casein feststellten, neben Trypsin auch Chymotrypsin. Das Mengenverhältnis der beiden Enzyme in jenem Proteinasepräparat ist nicht angegeben worden, doch glauben wir aus den Ergebnissen der von Waldschmidt-Leitz und Akabori angestellten Versuche über die Caseinspaltung durch Proteinase, Trypsin und eine Mischung von Trypsin und Chymotrypsin [[1]), Fig. 1, Tab. 2, 3, 4, 6] schließen zu können, daß die Menge des in der Proteinase enthaltenen Chymotrypsins recht beträchtlich gewesen sein dürfte. Die Mischung nämlich, mit der diese Autoren denselben Spaltungsbereich erzielten wie mit ihrem Proteinasepräparat, enthielt Trypsin und Chymotrypsin im Verhältnis 1:2,5. Hieraus darf nun zwar nicht der Schluß gezogen werden, daß das Proteinasepräparat die beiden Enzyme im selben Verhältnis enthalten haben müsse, da es sich ja nicht um die Messung von Spaltungsgeschwindigkeiten, sondern um die von Spaltungsbereichen handelt; aber einigermaßen scheint doch auch der Spaltungsbereich vom Mengenverhältnis der beiden Komponenten abzuhängen. Andernfalls hätte nämlich der Bereich der Caseinspaltung durch das von Waldschmidt-Leitz und Akabori untersuchte Trypsinpräparat nicht so wesentlich kleiner sein können als der von Proteinase, bzw. Trypsin + Chymotrypsin, da auch das Trypsinpräparat zweifellos eine, wenn auch wesentlich geringere (vermutlich von gleicher Größenordnung wie die Trypsinpräparate b und c der vorliegenden Untersuchung) Beimengung von Chymotrypsin enthalten hat.

Wir glauben daher nicht, daß das abweichende Verhalten des von uns untersuchten Proteinasepräparates durch dessen Gehalt an Chymotrypsin (Mengenverhältnis Trypsin: Chymotrypsin ungefähr 1,2:1) erklärt werden kann.

VERSUCHE

1. Substrat. Die vorliegende Probe von Clupeansulfat hatte einen NH_2-Quotienten von 89 (Angabe von E. Waldschmidt-Leitz). Sie wurde zu einer 1,25%igen Lösung in Wasser gelöst, welche nach dem Abfiltrieren eines geringen unlöslichen Rückstandes 2,45 mg N im ccm enthielt.

2. Enzympräparate. a) Proteinase. Klare Glycerinlösung, enthaltend nach Angabe 1 T.-(e.) pro Kubikzentimeter. Bei der Aktivitätsbestimmung mit Casein ergab 1 ccm, mit Enterokinase aktiviert, in 20 Minuten bei 30° eine

TABELLE 1

Enzympräparat	a		b		c		d	
	Zeit in Stdn.	Spaltung*)	Zeit in Stdn.	Spaltung	Zeit in Stdn.	Spaltung	Zeit in Stdn.	Spaltung
	0,03	0,32	0,05	0,52	0,05	0,58	0,03	0,32
	0,62	0,90	0,52	0,95	0,50	0,98	0,62	0,94
	3,2	1,06	3,2	1,04	3,2	1,01	3,2	1,01
	6,3	1,08	6,3	1,19	5,9	1,14	6,3	1,09
	22,5	1,27	23,0	1,27	22,5	1,34	22,5	1,23
nach neuerlichem...........	1,7	0,00	1,1	0,08	1,0	0,06	3,5	0,14
Enzymzusatz...............	24,0	0,16	13,0	0,17	12,0	0,06	24,0	0,14

*) mg Amino-N pro 0,05 gm Clupeansulfat.

Spaltung von 1,02 ccm 0,2 n-KOH (Alkoholtitration), was der obigen Angabe recht genau entspricht. Nach der Hämoglobinmethode[5]) bestimmt, enthielt 1 ccm 0,028 $[\text{T. U.}]^{\text{Hb}}$; die spezifische Aktivität war 0,107, da 1 ccm 0,261 mg Protein-N enthielt [bestimmt nach[6])]. Die durch Milchgerinnung bestimmte Chymotrypsinaktivität[7]) war 0,5 [R. U.] pro Kubikzentimeter, entsprechend einer spezifischen Aktivität von 1,9. Zum Vergleich seien hier die entsprechenden spezifischen Aktivitäten für krystallisierte Präparate von Trypsin ($([\text{T. U.})]^{\text{Hb}}_{\text{mg P.N.}}$ = 0,17) und Chymotrypsin ($([\text{R. U.}]_{\text{mg P.N.}}$ = 8,5) angeführt[8]).

b) Lösung von krystallisiertem Trypsin „Nr. 6". Dargestellt nach[2]) (vgl. dort Tab. IV) im September 1933. Spezifische Aktivität: $[\text{T. U.}]^{\text{Hb}}_{\text{mg P.N.}}$ = 0,096, $[\text{R. U.}]_{\text{mg P.N.}}$ = 0,065.

c) Lösung von 1 mal krystallisiertem Trypsin. Dargestellt nach[4]). Spezifische Aktivität: $[\text{T. U.}]^{\text{Hb}}_{\text{mg P.N.}}$ = 0,090, $[\text{R.U.}]_{\text{mg P.N.}}$ = 0,092.

d) Lösung von 6 mal umkrystallisiertem Trypsin. Dargestellt aus Präparat c). Spezifische Aktivität: $[T. U.]^{Hb}_{mg\ P.N.} = 0{,}15$; $[R. U.]_{mg.\ P.N.}$ nicht meßbar.

3. Spaltungsansatz. Alle Enzymlösungen wurden so verdünnt, daß für gleiche Clupeanmengen gleiche Enzymmengen, ausgedrückt in $[T. U.]^{Hb}$ zur Anwendung kamen. Die schließliche Zusammensetzung der Ansätze war:

1% ig an Clupeansulfat = 1,96 mg N/ccm,
0,04 molar an Phosphatpuffer (p_H = 7,7),
1 ccm = 0,0013 $[T. U.]^{Hb}$, entsprechend 0,043 T.-(e.)
Toluol als Antisepticum.

Die Reaktionstemperatur war 35,5°; die Bestimmung der Spaltung geschah im Mikroapparat nach van Slyke.

Als Nullpunkt für die Spaltungsbestimmungen diente der NH_2-Wert der enzymfreien Clupeansulfatlösung. Der mit den Enzymlösungen eingeführte NH_2-Betrag konnte, wie Kontrollbestimmungen zeigten, vernachlässigt werden.

Zur Prüfung der Vollständigkeit der Spaltung wurden nach 24 Stunden zu 7,5 ccm der Ansätze jeweils 3 ccm Enzymlösung, enthaltend 0,0032 $[T. U.]^{Hb}$ pro Kubikzentimeter, zugefügt.

Die Tabelle gibt die Spaltungen in mg NH_2-Stickstoff, umgerechnet auf eine Analysenprobe von 5,0 ccm, mit der von Waldschmidt-Leitz und Akabori angewendeten Substratkonzentration, welche $\frac{5{,}06}{4{,}90} = 1{,}03$ mal größer war, als bei unseren Versuchen. Diese Werte sind mit den Angaben der Tab. 13 und 14 der Abhandlung[1]) unmittelbar vergleichbar.

ZUSAMMENFASSUNG

Bei einem Vergleich der Spaltung von Clupeansulfat durch adsorptiv gereinigte Proteinase und verschiedene Präparate von krystallisiertem Trypsin ergaben sich gleiche Spaltungsbereiche.

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ACETYLATION OF TYROSINE IN PEPSIN

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In an earlier paper (1) the writer described the preparation and isolation of three crystalline acetyl derivatives of pepsin. They were, "100 per cent active" acetyl pepsin in which the 3 or 4 primary amino groups of pepsin had been acetylated with less than 15 per cent change in the specific activity; "60 per cent active" acetyl pepsin which contained 6–11 acetyl groups per molecule; and "10 per cent active" acetyl pepsin which had 20–30 acetyl groups per molecule of protein. Reversion of the 60 per cent active enzyme into the 100 per cent active was effected by treatment with normal sulfuric acid at 5°C.[1] It was pointed out in this earlier work that in all probability the primary amino groups belong to the lysine part of the protein molecule and that since acetylation of these groups failed to produce any appreciable effect on the activity of pepsin that the rôle played by the lysine in the enzymatic activity of the molecule is probably relatively small.

Since the introduction of a few acetyl radicals into other groups of the protein molecule definitely diminished the specific activity, it was reasoned that these groups of the protein must be more closely related to the seat of the enzymatic activity.

The present work was undertaken to determine the structural position of these few acetyl groups which have such a pronounced effect on the activity of pepsin. Though the evidence is somewhat indirect it nevertheless seems probable that those acetyl groups in the 60 per cent active acetyl pepsin which are responsible for the decrease in specific enzymatic activity are attached to the phenolic hydroxyl groups of some of the tyrosine components of the protein.

[1] In the previous paper, *J. Gen. Physiol.*, 1934, **18**, 53, 54, the temperature is given as 10°C. This should read 5°C.

In the 60 per cent active preparation there are three acetyl groups which are not in the 100 per cent active acetyl pepsin. These three additional acetyl groups are rapidly hydrolyzed by molar acid or by alkali at pH 10.0, whereas the acetyl groups on the amino groups are not hydrolyzed under the same conditions. With this property in mind these easily hydrolyzed acetyl groups have been designated as "pH 10.0 labile" acetyl groups. There are three less tyrosine phenol groups in the 60 per cent active acetyl pepsin as measured colorimetrically with the Folin phenol reagent under conditions which will not hydrolyze an acetylated phenol.

When the 60 per cent active material is changed back into 100 per cent active acetyl pepsin by the above mentioned acid treatment there is an accompanying loss of the pH 10.0 labile acetyl groups and the number of tyrosine phenol groups returns to that of the original pepsin.

EXPERIMENTAL RESULTS

Preparation.—A slight modification in the method of preparation of the 60 per cent active acetyl pepsin has led to more uniform and reproducible results. Table I contains the analyses of several different acetyl derivatives and of pepsin. In this table the 100 per cent active acetyl pepsin was prepared by acid hydrolysis of the 60 per cent active materials. A discussion of the procedures and the interpretation of the analyses will be found later in this paper but it might be noted at this time that the acetyl group figures are more significant than the tyrosine phenol group figures for the determination of the former is subject to less error and the interpretation of the figures is on a more sound chemical basis.

From Table I it appears that with the change from pepsin to 60 per cent active acetyl pepsin there is an increase of three pH 10.0 labile acetyl groups (besides the increase of 3 or 4 acetyl groups on the primary amino groups). There is also a decrease in the tyrosine-tryptophane value of the protein as measured by the colorimetric "pH 8.0 method," equivalent to 3 tyrosine phenol groups.

Experimental Procedure

In general the materials, the analyses of which appear in Table I, were prepared by the methods described previously (1). In the present instance, however, the

60 per cent active acetyl pepsin was prepared in the same way that was previously described for the preparation of 10 per cent active acetyl pepsin except that the acetylation by ketene was stopped when the specific activity was approximately 60 per cent of the original pepsin. This point was determined by analyzing samples taken from time to time. The material was then precipitated from solution by acidification and half saturation with magnesium sulfate, fractionated, crystallized, and finally dialyzed in collodion bags for 24 hours on a dialyzer (2)

TABLE I

Acetyl and Tyrosine Analyses of Pepsin and Its Acetyl Derivatives

Name	No.	$[\text{P. U.}]^{\text{Hb}}_{\text{mg. P. N.}}$	Tyrosine-tryptophane content by colorimetric method*					No. of acetyl groups per mol pepsin	
			Per cent protein		No. of groups per mol pepsin				
			pH 11.0 method	pH 8.0 method	pH 11.0 method	pH 8.0 method	(pH 11–pH 8)	pH 10 labile	Total
3 x cryst. P. D. pepsin	1	0.21	11.8	11.8	24	24	0	0.0	1
5 x " " " "		0.20	11.7	11.7	24	24	0	0.0	
100 per cent active acetyl pepsin†	20	0.17	12.0	11.5	24	23	1	0.2	
" " " " " " †	24	0.18	11.7	12.0	24	24	0		
" " " " " " ‡	11	0.18						0.3	3
60 per cent active acetyl pepsin†	11	0.12	11.8	10.4	24	21	3	3.6	7
" " " " " " †	16	0.12	12.0	10.7	24	21	3	3.0 ± 0.2	6
10 per cent active acetyl pepsin§	6	0.037	12.0	8.2	24	16	8	14	18
" " " " " " §	6	0.036	12.2	7.7	24	15	9	13	16

* See discussion under Experimental methods for the determination and calculation of these figures.

† Crystallized and fractionated.

‡ Reaction mixture; *i.e.*, not fractionated.

§ Fractionated but not crystallized.

at 5°C. against M/2000 pH 4.65 acetate buffer. All preparations and samples were dialyzed as just described before final analysis. The 100 per cent active acetyl pepsin preparations were made by subjecting the 60 per cent active preparations to 1.25 N sulfuric acid at 5°C. for 75–100 hours followed by concentration, fractionation, crystallization, and dialysis. The activity estimation was made by the hemoglobin method of Anson and Mirsky (3). The other analyses were carried out as described under Experimental methods.

On treatment of the 60 per cent active acetyl pepsin with normal sulfuric acid at 5°C. the specific enzymatic activity of the protein

rises to that of pepsin.[2] *The pH 10.0 labile acetyl groups are no longer detectable and the tyrosine-tryptophane value has increased* to that of pepsin. It will be shown later that it is highly probable that these changes in tyrosine-tryptophane value are due to the coupling or hydrolysis of acetyl groups on tyrosine phenol groups of the protein. It is concluded, therefore, that the change in specific activity from 100 per cent active acetyl pepsin to 60 per cent active acetyl pepsin can be attributed to the acetylation of 3 tyrosine phenol groups in the protein. It is possible that not all 3 of these tyrosine phenol groups are involved in the effect produced on the activity but the writer has endeavored without success to obtain decisive evidence on this point.

In the change from 60 per cent active acetyl pepsin to the 10 per cent active acetyl pepsin there is a further increase in pH 10.0 labile acetyl groups and a decrease in tyrosine-tryptophane value of the protein. The change in the number of acetyl groups, however, is not equivalent to the decrease in number of tyrosine phenol groups calculated from the chromogenic value so that some of these acetyl groups may be attached to other than tyrosine phenol groups.

Tyrosine Content of Pepsin

It was pointed out by Wu (4) that the color produced by the phenol reagent in the presence of proteins is largely due to the tyrosine in the protein. Since two other amino acids, tryptophane (5) and cysteine (6) produce the characteristic blue color with the phenol reagent they must be considered as possible sources of color when a protein is treated with alkali and the phenol reagent. There is also the possibility that there exists in some proteins a component other than the amino acids and that this component will reduce the phenol reagent. Heme of hemoglobin is such a component and is known to reduce the reagent (6). Amino acids which in the pure state do not produce the color with the reagents may, when in combination with

[2] The figures given for the specific activity of the 100 per cent active acetyl pepsin are some 10–20 per cent below that of pepsin. Preparations have been obtained with a specific activity more nearly that of pepsin ($[\mathrm{P.U.}]^{\mathrm{Hb}}_{\mathrm{mg.\,P.N.}}$ = 0.20–0.22) but a small fraction of these preparations seems to be unstable with respect to activity and is lost on standing; the result being a lowering in the specific activity of the total material.

other amino acids in the protein, then have the property of reducing the color reagents. *There is no evidence for these possibilities in the* case of pepsin except for one observation,[3] and there seems to be no correlation of this with the present work. Pepsin gives a negative nitro-prusside test for free S H groups. Cysteine is, therefore, probably not present and the color giving property of pepsin is due to the tyrosine and tryptophane.

The estimation of the tyrosine-tryptophane content of pepsin was carried out in the present instance by two related colorimetric methods in which Folin's phenol reagent (7) was used. The conditions of one method, designated as the pH 8.0 method, are so arranged that free tyrosine phenol groups may be determined in the presence of, but without the hydrolysis of, acetylated phenol groups. The conditions of the other method, designated as the "pH 11.0 method," are such as to hydrolyze the acetylated phenols and then measure the total number of phenol groups with the same reagents and under the same conditions as the pH 8.0 method employs. From these two methods the total[4] (free plus acetylated) phenol groups and the free phenol groups are determined. The difference between the two designates the number of acetylated groups. A discussion and an outline of the procedures are to be found in the section devoted to Experimental methods.

Rate of Hydrolysis of pH 10.0 Labile Acetyl Groups and Diacetyl Tyrosine in Acid

It was brought out in our earlier work (1) that there is a difference in some of the acetyl groups of 60 per cent active acetylated pepsin with respect to acid hydrolysis. Those which are attached to the primary amino groups are hydrolyzed by acid with comparative diffi-

[3] When to solutions of pepsin or other proteins of such a concentration as is used in measuring the chromogenic value by phenol reagents, is added 1 ml. of 0.002–0.0005 M $CuSO_4$ the chromogenic value is increased from 1–3 times. This increase in color value is not demonstrable with acid hydrolysates of pepsin though it is with enzymatic hydrolysates. The increase is very noticeable on purified gelatin which, according to the accepted analyses, contains little or no tyrosine and tryptophane. Proline or a pyrolle type component is suspected though in the pure state and in the presence of $CuSO_4$ proline and hydroxy proline show no action toward the phenol reagents.

culty. Fig. 1 shows graphically the results of an experiment in which a solution of diacetyl tyrosine, prepared as directed by Bergmann and Stern (8), and a solution of 60 per cent active acetyl pepsin were hydrolyzed by 1.25 N sulfuric acid at 5°C. Experimental difficulties prevented measurement of the tyrosine-tryptophane color value and the acetyl estimation during the hydrolysis of the enzyme solution. The pH 10.0 labile acetyl analysis was, however, performed on the initial and final products and is included in Fig. 1. The experiment

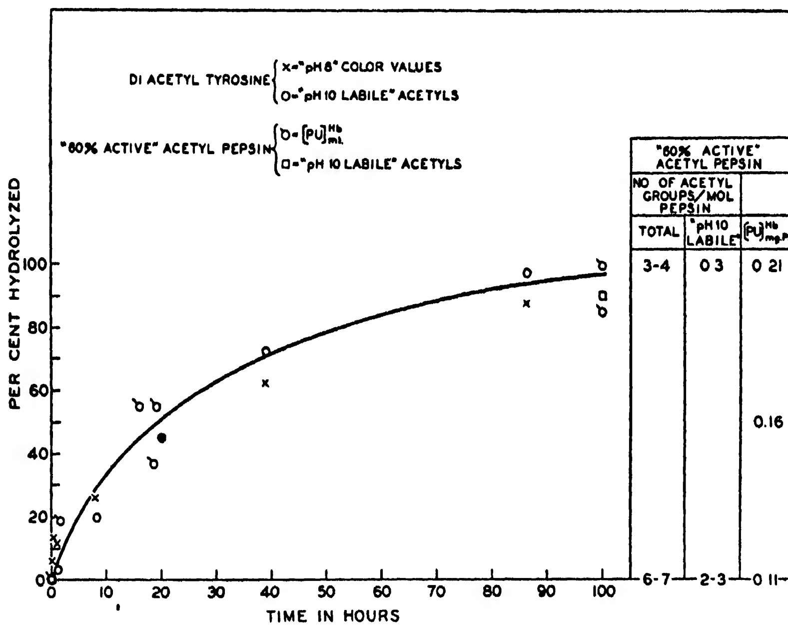

FIG. 1. Rate of hydrolysis of 60 per cent active acetyl pepsin and of diacetyl tyrosine in 1.25 N sulfuric acid at 5°C.

confirms the experiments of our previous paper in which it was demonstrated that in strong acid the specific activity of the enzyme returns to approximately that of pepsin and the acetylated protein loses some of its acetyl groups. In addition it shows that the rate at which this reactivation takes place is very close to the rate at which the acetyl group on the phenol group of diacetyl tyrosine is hydrolyzed under identical conditions.

Experimental Procedure

Enzyme.—250 ml. of a dialyzed preparation of 60 per cent active acetyl pepsin containing 3.0 mg. P.N./ml. was cooled to 5°C., added to 250 ml. of cooled 2.5 N sulfuric acid, and the suspension stirred continuously. Under these conditions a large part of the protein is insoluble. Samples were taken from time to time, the precipitate filtered off, and the acid filtrate neutralized with an equal volume of 1.5 N sodium acetate. Analyses for protein nitrogen and enzymatic activity were made on this neutralized filtrate. When the specific activity had reached that of pepsin the total protein was precipitated, fractionated, crystallized, and analyzed for specific activity, pH 10.0 labile, and total acetyl groups. A sample of the original 60 per cent active pepsin was analyzed at the same time.

Diacetyl Tyrosine.—0.5 gm. of crystalline diacetyl tyrosine was dissolved in 79 ml. water with the aid of 1 ml. of M/1 pH 5.0 citrate buffer. 70 ml. of this solution was cooled to 5°C. and added with stirring to 70 ml. of 2.5 N sulfuric acid at 5°C. 20 ml. samples were taken from time to time and analyzed for free acetic acid by distillation from a 3 molar citrate buffer pH 4.0 and subsequent titration of the distillate. Samples were also analyzed for free phenol groups by the pH 8.0 method. In the early part of the hydrolysis the measurement of free phenol groups was made possible by adding known quantities of tyrosine to the aliquot of reaction mixture being analyzed by the pH 8.0 method and then corrected for in the calculation. This reduced the error due to comparison of widely different colorimeter readings.

Rate of Hydrolysis of pH 10.0 Labile Acetyl Groups and of Diacetyl Tyrosine in Alkali

Acetyl groups may be hydrolyzed by alkali as well as by acid and so an experiment was performed at pH 9.0–10.0 to see if the acetyl group on the phenol group of tyrosine is hydrolyzed under the conditions which hydrolyze those on 60 per cent active acetyl pepsin.[4] The results are shown in Fig. 2. Unfortunately the pH of the medium was not exactly the same for the two materials, that of the protein solution being pH 9.8, compared to pH 9.0 of the diacetyl tyrosine solution (pH measurements by hydrogen electrode). Since the enzyme is immediately inactivated under these conditions the activity

[4] In the medium for hydrolysis, pH 9.0–11.0, of the labile acetyl groups a glycine buffer and probably any material containing free amino groups should be avoided in high concentrations (0.2 molar or greater) for it was found with glycine that the amino group acts as an acceptor of the liberated acetyl group after hydrolysis. It is, therefore, not free to be estimated as acetic acid and is hydrolyzed from glycine only after relatively vigorous hydrolytic treatment.

could not be followed. The change in the tyrosine-tryptophane value of the protein by the pH 8.0 method was followed along with the change in acetyl groups. It seems perfectly clear from Fig. 2 that at pH 9.0–10.0 the hydrolysis of acetyl groups from 60 per cent active acetyl pepsin is accompanied by a corresponding increase in the pH 8.0 chromogenic value of the protein. Also that the hydrolysis of the oxygen acetyl linkage of diacetyl tyrosine takes place at approximately

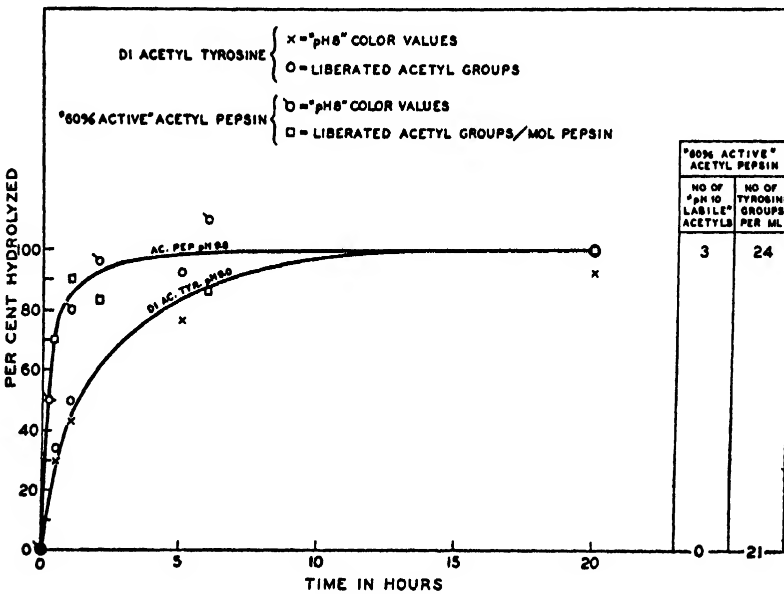

FIG. 2. Rate of hydrolysis of 60 per cent active acetyl pepsin and diacetyl tyrosine at pH 9.0–10.0 and 35°C.

the same rate as does the hydrolysis of the pH 10.0 labile acetyl groups of the above mentioned 60 per cent active acetyl pepsin.

Experimental Procedure

Enzyme.—To 41 ml. of 60 per cent active acetyl pepsin containing 13 mg. P.N./ml. was added with stirring a solution of 5.2 ml. 1 N sodium hydroxide and 23.8 ml. M/10 borate buffer pH 10.5, final pH 9.8 determined by the hydrogen electrode. The solution was kept at 35.5°C. throughout the experiment. Samples were taken at varying intervals of time and analyzed for free acetic acid by distillation from 3 M citric acid with subsequent titration of the distillate. Analyses were also made for free phenol groups by the pH 8.0 method.

Diacetyl Tyrosine.—To 0.4 gm. of crystalline diacetyl tyrosine was added 35 ml.

of water containing 0.5 ml. M/1 pH 5.0 citrate buffer. To this solution was added with stirring 34 ml. M/10 pH 10.5 borate buffer and 1 ml. of 1 N sodium hydroxide, the resulting mixture being pH 9.0 by the hydrogen electrode. Aliquots were analyzed from time to time for free acetic acid and free phenol groups by the above mentioned procedures. The reaction mixture was also kept at 35.5°C.

Rate of Acetylation of Tyrosine and Tryptophane

It seems highly probable from the foregoing experiments that the groups in the protein pepsin which, when acetylated, are responsible for the changes in activity and pH 8.0 chromogenic value, are tyrosine phenol groups. There exists, however, the possibility that the color producing group or structure of tryptophane in the protein pepsin may be acetylated and, when acetylated, causes the change in specific enzymatic activity. It was thought that a comparison of the rate of acetylation of tyrosine and of tryptophane would furnish evidence in this connection. It was decided to attempt acetylation under the conditions used for the acetylation of pepsin; *i.e.*, in strong acetate buffer (pH 5.0–6.0). Since tyrosine is only slightly soluble under these conditions the glycyl derivatives of the two amino acids were used instead. Under the conditions of acetylation (in strong acetate buffer) acetyl figures would have been very difficult. Following the change in chromogenic value of the solution by both the pH 11.0 and pH 8.0 methods serves much the same purpose. By comparing the color values obtained by these two methods one may determine quantitatively the alkali reversible change in color properties of the molecule due to acetylation.

Experimental Procedure

0.5–1.0 gm. of Hoffman-La Roche preparations were dissolved or suspended in approximately 40 ml. of 3 M acetate buffer at the pH indicated. Ketene from the generator previously described (9) and which was used in the preparation of the acetyl pepsin derivatives was passed in slowly with stirring. The materials, if only slightly soluble in the initial stage, were quickly converted (probably by acetylation of the amino groups) into a soluble form. Aliquots were removed at varying intervals of time, diluted, and chromogenic values determined by the pH 8.0 and pH 11.0 methods.

From Table II it is readily seen that there is a gradual decrease in pH 8.0 color value of glycyl tyrosine solution during acetylation, whereas the pH 11.0 color value remains constant. This is inter-

preted as acetylation of the phenol group of glycyl tyrosine. The acetyl group is hydrolyzed yielding the full value in the pH 11.0 method, whereas the pH 8.0 method measures only the free or unacetylated phenol groups. In the case of tryptophane and glycyl tryptophane there is a decrease in color values as measured by both

TABLE II

Acetylation of Glycyl Tyrosine, Glycyl Tryptophane, and Tryptophane by Ketene

Material	pH	Time	Calculated from color value by the		Ratio pH 8.0 value to pH 11.0 value	Acetylated (calculated)
			pH 11.0 method	pH 8.0 method		
		hrs.	*mg./ml.*	*mg./ml.*		*per cent*
		0	1.6	1.5	0.94	0
		0.5	1.6	1.2	0.75	20
		1.0	1.6	0.92	0.57	39
Glycyl tyrosine	5–6	1.5	1.5	0.57	0.38	60
		2.0	1.5	0.47	0.31	67
		3.0	1.4	0.28	0.20	79
		0	2.0	1.2	0.60	0
		1.0	1.2	0.76	0.63	0
Glycyl tryptophane	5–6	2.0	1.1	0.75	0.68	0
		5.0	1.2	0.90	0.73	0
		0	3.7	3.9	1.1	0
Tryptophane	5–6	2.0	2.3	2.8	1.2	0
		4.0	2.1	2.0	1.0	0
		0	1.7	1.6	0.94	0
		0.5	1.6	1.4	0.87	7
	4.0	1.0	1.7	1.3	0.77	18
Glycyl tyrosine	—	1.5	1.7	1.2	0.71	24
	4.3	2.0	1.6	1.1	0.69	26
		3.0	1.6	1.0	0.63	33

methods. The ratio of pH 8.0 color to pH 11.0 color is practically constant throughout although there is a considerable loss in total color value. The tryptophane solution after 4 hours of acetylation was heated with alkali and yet the change in color value occurring during acetylation did not revert to its original value. The change in color of tryptophane and glycyl tryptophane, whether caused by

acetylation or some other factor, is therefore entirely different from the change which takes place on acetylation of glycyl tyrosine or of pepsin.

In the previous paper (1) it was pointed out that the specific activity of pepsin drops much more slowly when acetylation is carried out at pH 4.0–4.5 than when carried out at pH 5.0–6.0. If, then, the change in specific activity during acetylation of pepsin is due to acetylation of the tyrosine phenol group, it might be expected that the phenol group of pure tyrosine or glycyl tyrosine would acetylate more slowly at pH 4.0–4.5 than at pH 5.0–6.0. This was found to be the case as may be seen in Table II. The rate at pH 4.3 is less than one-half that at pH 5.6. The results of these two experiments tend to eliminate the possibility of tryptophane and point definitely to tyrosine as being the component of pepsin which, when acetylated, results in a marked decrease in specific activity.

Action of Hydrolytic Enzymes on Acetyl Derivatives of Pepsin

All of the foregoing proofs for the existence of acetylated phenol groups in acetylated derivatives of pepsin have been indirect or by analogy. In hope for a more direct proof an attempt was made to isolate the acetylated tyrosine from the acetyl pepsin derivatives. Since acid and alkali will hydrolyze acetylated phenols it was decided to use enzyme solutions as the hydrolytic agents. Solutions of 10 per cent active, 60 per cent active acetyl pepsins, and pepsin (control) as substrates were hydrolyzed at pH 7.0–7.5 with Fairchild's crude trypsin, Wilson's commercial steapsin, crystalline trypsin and chymotrypsin; with the same enzyme solutions at pH 6.0 after heat denaturation of the pepsins; and with Parke Davis 1:10,000 pepsin and crystalline pepsin at pH 2.0 after denaturation; and with the preceding enzymes at pH 6.0 in the native state. During the experiments the chromogenic values of the solutions were followed by the pH 11.0 and pH 8.0 methods and in the last instance ("native" pepsin and acetyl derivatives at pH 6.0) the specific enzymatic activity was followed. Hydrolysis by Fairchild's trypsin of the three different enzyme preparations was attempted at 5°C., 20–25°C. (room temperature), and 35°C.

The results were completely negative with regard to isolating any

acetyl tyrosine from the acetylated pepsins. Fairchild's trypsin carries the digestion far enough to release the tyrosine molecule but it also contains some material which brings about the hydrolysis of the acetyl group of the oxy-acetyl tyrosine. The action of the tryptic enzymes on native acetyl pepsins in no case produced an increase in specific activity of the enzyme and no increase in pH 8.0 chromogenic value of the proteins although there was destruction of a large part of the total protein. It seems probable, therefore, that the agent which brings about hydrolysis of oxy-acetyl tyrosine in the decomposition products of the acetyl derivatives of pepsin will not act similarly on the native active protein.

EXPERIMENTAL METHODS

In general the technique in handling the proteins, measuring enzymatic activity, nitrogen, pH, etc. was the same as that used and described in the previous work (1). Any deviations or new procedures are described below or in the experimental procedures found in the main body of this paper.

Acetyl Estimation

Total Acetyls.—The procedure for estimating acetyl groups has been improved over that described in the previous paper so that a detailed account of the procedure will be given. All protein solutions to be analyzed for acetyls were dialyzed in a Kunitz and Simms (2) dialyzer for at least 20 hours at 5°C. against M/2000 pH 4.65 acetate buffer. This reduces the acetate ion of a protein solution to approximately M/2000 which is negligibly small. Dialysis also removes any buffers, salts, and most of the non-protein nitrogen. From a nitrogen analysis of this dialyzed solution the protein content is calculated, assuming the pepsin to contain 15.4 per cent nitrogen. To a volume containing approximately 0.5 gm. of protein is then added 1 ml. of 4.2 N sodium hydroxide and the volume, if less than 12 ml., is diluted with water to 12 ml. and placed at 35.5°C. for 5 days. After this time the entire volume is mixed with 5 ml. of 3.5 M citric acid and 0.1–0.2 ml. octyl alcohol in a 150 ml. modified Claissen distilling flask and distilled for approximately 15 minutes at 50°C. ± 5°C. and 20 mm. pressure. After the distillation a drop of 0.5 per cent phenolphthalein is added to the receiving flask and the distillate titrated with N/50 sodium hydroxide and the titration checked by adding a drop of 0.1 per cent brom cresol green and back titrating to pH 4.7 with N/50 hydrochloric acid. The latter or back, titration will be half the first titration if the acid in the distillate is acetic acid. After this titration the receiving flask is rinsed out with distilled water, 10–15 ml. of distilled water and 0.1–0.2 ml. octyl alcohol added to the distilling flask, and the distillation and titration repeated. This procedure is repeated three or four times or until the titration of the distillate is

less than 0.25 ml. of N/50 sodium hydroxide. A blank titration of 0.10 ml. N/50 sodium hydroxide is subtracted from each titration and the acetyls calculated from the sum of the titrations.

pH 10.0 Labile Acetyls.—The protein solutions were first freed of soluble non-protein nitrogen, acetate ion, salts, etc. by dialysis just as in the above procedure. From a nitrogen analysis the protein/ml. is calculated and a volume is then taken which contains approximately 0.5 gm. of protein. To this is added with stirring a mixture of 5 ml. of saturated borax and 1 ml. of N/1 sodium hydroxide. If 0.5 gm. of pepsin protein is taken and if the non-protein nitrogen content is not above 10 per cent of the total nitrogen the pH of the solution resulting from the above mixture will be pH 9.8 ± 0.2. This solution is allowed to stand at 35.5°C. for approximately 20 hours after which time the acetic acid is distilled and estimated just as described in the estimation of total acetyls.

Determination of Tyrosine Phenol Groups

The tyrosine phenol groups of pepsin and of its acetyl derivatives were estimated colorimetrically by means of Folin's phenol reagent (7) under particular conditions with reference to pH. The alkali reagent is made up so that the final solution in which the color develops is pH 8.0. At this pH tyrosine phenol groups give rise to color with the phenol reagent but acetylated tyrosine phenol groups do not.[5] This procedure has been called the pH 8.0 method and the details of it are to be found later in this section.

The procedure designated as the pH 11.0 method differs from the pH 8.0 method only in that the sample to be analyzed is made alkaline to pH 11.0–12.0 then adjusted to pH 8.0 for estimation of the color value. This treatment hydrolyzes all acetylated tyrosine phenol groups and gives, therefore, the total tyrosine color value of the sample.

The pH 8.0 method was standardized by determining the tyrosine and tryptophane content of several different proteins by the colorimetric method at pH 8.0 and comparing these values with tyrosine and tryptophane analyses obtained by other methods. These determinations are to be found in Table III. Unfortunately many proteins are insoluble under the conditions necessary for this measurement but Table III includes analyses of four proteins with different tyrosine-tryptophane values which are soluble at pH 8.0 in the presence of the phenol reagents. On the average 59 per cent of the color expected from the tyrosine and tryptophane content of these four proteins was found by the pH 8.0 method. From this fact it appears that the tyrosine and tryptophane groups of proteins do not give their full color value by the pH 8.0 method. It is possible, of course, that

[5] This property is not a peculiarity of the *tyrosine* phenol group for almost any phenol group gives rise to the characteristic blue color with the phenol reagent (10). The writer has found that no color was obtained by the pH 8.0 method when phenyl acetate or acetyl salicylic acid were used whereas they give the expected quantity of color by the pH 11.0 method.

only 59 per cent of the total number of these groups in protein react at pH 8.0 but with several proteins containing different amounts of tyrosine and tryptophane this interpretation seems unlikely. As yet the writer has no decisive experimental evidence on the question. The interpretation, however, affects only the exact value and does not change the order of magnitude of the final figure.

Amino acid analyses of crystalline pepsin show about 10.3 per cent tyrosine and 2.2 per cent tryptophane.[6] In order to have a common basis for color giving groups the tryptophane value may be expressed in terms of tyrosine. Thus, 2.2 per cent tryptophane is equivalent to about 2.0 per cent tyrosine. The tyrosine-tryptophane content of pepsin, expressed in terms of tyrosine is, therefore, 12.3

TABLE III

Tyrosine-Tryptophane Content of Several Proteins as Determined by the pH 8.0 Colorimetric Method and the Usual Method after Acid Hydrolysis

Protein	pH 8.0 method	Analysis after acid hydrolysis: Tyrosine	Analysis after acid hydrolysis: Tryptophane	Analysis after acid hydrolysis: Tyrosine + tryptophane expressed as tyrosine	Ratio: Tyrosine + tryptophane content by pH 8.0 method / Tyrosine + tryptophane content after hydrolysis
	per cent	*per cent*	*per cent*	*per cent*	
Dialyzed 5 x cryst. P.D. pepsin	7.9	10.3*	2.2*	12.3	0.64
Dialyzed 3 x cryst. egg albumin	2.9	4.0†	1.2†	5.1	0.57
Kahlbaum-Hammarsten casein	4.1	6.4†	1.4†	7.6	0.54
Horse serum albumin	3.0	4.7†	0.5†	5.1	0.59
					Average 0.59 ± 0.03

* Personal communication of Dr. H. O. Calvery.

† Analyses by Folin and associates.

per cent or 25 tyrosine groups per mole of pepsin. The tyrosine-tryptophane content of pepsin, as determined by the pH 8.0 method is about 7.7 per cent. Assuming that all the groups react under these conditions, one mol of tyrosine or tryptophane in pepsin gives the color equivalent of $\frac{7.7}{12.3} = 0.64$ mols of tyrosine. In determining the tyrosine and tryptophane content of pepsin at pH 8.0, therefore, the total tyrosine-tryptophane is calculated as $\frac{1.0}{0.64} = 1.6$ times the quantity

[6] A personal communication from Dr. H. O. Calvery of the Department of Physiological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan.

of tyrosine which develops the same color as the pepsin. The results are shown in Table I.

It may be seen in Table I that the 10 per cent active and 60 per cent active acetyl pepsins have tyrosine-tryptophane values by the pH 8.0 method less than that of pepsin. If these materials are all titrated to pH 11.0–12.0, left for a moment, and then acidified to pH 8.0 followed by an estimation of the color by the pH 8.0 method they will all show the same value as pepsin. This scheme has been used in this work and has been designated as the pH 11.0 method. The alkali

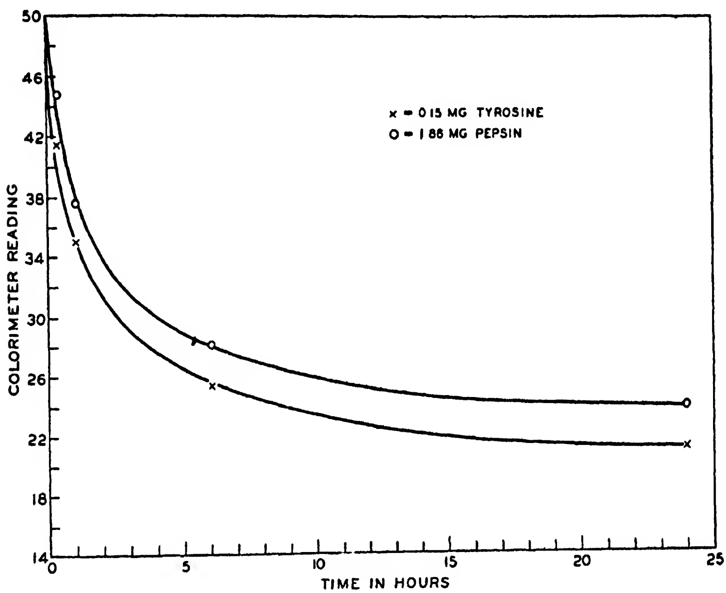

Fig. 3. Rate of color development of pepsin and pure tyrosine by pH 8.0 method.

at pH 11.0–12.0 quickly hydrolyzes the acetylated tyrosine phenol groups thus returning the number of pH 8.0 color giving groups to that of pepsin.

In both the pH 8.0 and pH 11.0 methods the phenol reagent is added to the protein solution before the alkaline buffer is added to prevent the solution ever getting above pH 8.0. The color develops slowly at pH 8.0 and reaches a maximum in 10–24 hours at room temperature. As may be seen from Fig. 3 the rate of color development is the same in the protein pepsin as in pure tyrosine. One is justified, therefore, in comparing the colors produced by the protein and by tyrosine at any arbitrary time interval as long as it is the same for both materials. A time interval of 15 minutes has been used during which time the flasks containing the colored solutions were kept at 35.5°C.

Experimental Procedure

The procedure used in the experiment, shown graphically in Fig. 3 was as follows: 1.86 mg. of purified pepsin and 0.15 mg. of tyrosine respectively were added to two 50 ml. Erlenmeyer flasks and diluted with water to a volume of 17 ml., followed by 3 ml. of 1/3 diluted Folin's phenol reagent and 5 ml. of the alkaline phosphate solution (60 ml. 0.5 M K_2HPO_4 + 34 ml. N/1 NaOH + 6 ml. H_2O). The alkaline phosphate was added to the flask with stirring by whirling the flask. The solutions were allowed to remain at room temperature and from time to time were read in the colorimeter against a standard blue glass.

pH 8.0 Method.—An amount of material yielding a colorimeter reading approximately equal to that produced by 0.30 mg. of tyrosine under similar conditions is diluted to 11 ml. with water. To this solution is added 6 ml. of N/10 sodium chloride solution and 3 ml. of a 1:3 dilution of Folin's phenol reagent followed by 5 ml. of an alkali phosphate. (The alkali phosphate solution is made up of 60 ml. of 0.5 M K_2HPO_4 + 34 ml. N/1 NaOH + 6 ml. of H_2O.) This solution is placed at 35.5°C. for 15 minutes and compared to a solution of 0.30 mg. of tyrosine under similar conditions. If the amount of salts, buffers, and non-protein nitrogen content of the original material to be tested is very small the pH of the final colored solution will be pH 7.8 ± 0.2. If any buffer or alkali neutralizing material be present to any appreciable extent (which should be determined before the estimation by simply titrating an aliquot) a determined amount of alkali should be added to the 5 ml. of alkali phosphate to bring the solution to the same pH in all measurements. Approximately 3.0 mg. of pepsin protein is used in the pH 8.0 method of estimating tyrosine-tryptophane values. Aliquots of a standard solution of tyrosine were run parallel to the protein solutions using the same technique and reagents.

pH 11.0 Method.—An amount of material yielding a colorimeter reading approximately equal to that produced by 0.30 mg. of tyrosine under similar conditions is diluted to 11 ml. with water. To this solution is added 3 ml. of N/10 sodium hydroxide and the solution allowed to stand about 5 minutes and then the alkali is neutralized by 3 ml. of N/10 hydrochloric acid. 3 ml. of 1:3 dilution of Folin's phenol reagent is added followed by the introduction of 5 ml. of the alkali phosphate solution described in the pH 8.0 method. The 3 ml. of M/10 sodium hydroxide is sufficiently strong (unless buffers are present) to carry the pH of the solution to or beyond pH 11.0 where the acetyl groups come off of the phenol groups almost instantaneously.[7]

[7] In some of the experiments reported in this paper the procedure of the pH 11.0 method was not identical with that described above. The color was allowed to develop at pH 11.0–12.0 rather than at pH 8.0. The results obtained in this way, although different from the results by the above described pH 11.0 method, were proportionately different for all the chromogenic materials used and so the end result was not affected. The pH 11.0 method, as above described, was later developed and is to be preferred because of its general convenience.

SUMMARY

Crystalline 60 per cent active acetyl pepsin has 7 acetyl groups per mol of pepsin, 3 of which are readily hydrolyzed in acid at pH 0.0 or in weak alkali at pH 10.0.

The tyrosine-tryptophane content of this acetylated pepsin, measured colorimetrically, is less than pepsin by three tyrosine equivalents.

Hydrolysis at pH 0.0 or pH 10.0 of the 3 acetyl groups results in a concomitant increase in the number of tyrosine equivalents. In the pH 0.0 hydrolysis experiment there is also a simultaneous increase in specific activity.

The phenol group of glycyl tyrosine is acetylated by ketene under the conditions used in the acetylation of pepsin and the effect of pH on the rate of acetylation is similar in the two cases.

It is concluded that the acetyl groups in the 60 per cent active acetyl pepsin, which are responsible for the decrease in specific enzymatic activity, are 3 in number and are attached to 3 tyrosine phenol groups of the pepsin molecule.

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PACEMAKERS IN NITELLA

I. TEMPORARY LOCAL DIFFERENCES IN RHYTHM

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(Accepted for publication, May 22, 1935)

Theoretically a pacemaker can be produced at any spot where the P.D. can be sufficiently reduced and a sharp potential gradient can be maintained. This can be done experimentally[1] by means of KCl, chloroform, or ethyl alcohol.

Pacemakers occur spontaneously in many cases and may produce a variety of rhythms.[2] In studying these we have observed a phenomenon which recalls a well known feature of auricular flutter in the heart; *i.e.*, when the auricle beats 300 times per minute the ventricle may beat at half that rate.[3] In this case every other negative variation of the auricle is registered in the ventricle.

The situation in *Nitella* is shown in Fig. 1. The cell was arranged as in Fig. 2. A series of negative variations, originating spontaneously from a pacemaker near the right end of the cell, passed in succession through *E*, *D*, and *C*. The negative variations at these spots are recorded by separate strings and are shown on the record at *C*, *D*, and *E*.[4]

The record begins at the point marked 1 with a monophasic negative variation shown by the upward and downward[5] movement of the action curve at *E*, followed by similar movements at *D* and *C*. After a second variation a break occurs at 2. At 3, two more move-

[1] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 673. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1930–31, **14**, 611.

[2] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1934–35, **18**, 499.

[3] Lewis, T., Clinical electrocardiography, London, Shaw and Sons, 1913, 84.

[4] Regarding material and technique see Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87; 1934–35, **18**, 499.

[5] See Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 377.

ments occur at *E*, followed by single movements at *D* and *C*. In the movements starting at 4, each movement at *E* is followed by one at *D* and *C*.

Such a state of affairs may evidently depend on a change in the refractory period. It has been suggested[6] that the refractory period depends largely on the time required to move back into the sap the potassium which has moved out during the action current. This in turn would depend on the permeability of the protoplasm and the forces producing the movement of potassium: these forces are, of course, derived from metabolism.[7]

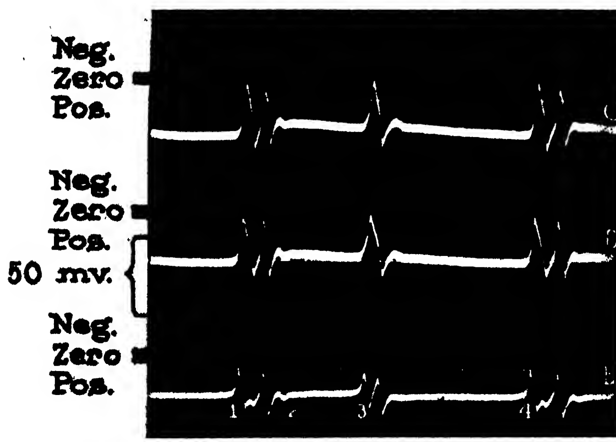

FIG. 1. Photographic record of negative variations, the cell being arranged as in Fig. 2.

A series of negative variations, originating near the right end of the cell, passed in succession through *E*, *D*, and *C* (Fig. 2). The variations at these spots are recorded by separate strings and are shown on the record at *C*, *D*, and *E*. The record begins at the point marked 1, with a monophasic negative variation shown by the upward and downward movement of the action curve at *E*, followed by similar movements at *D* and *C*. After a second variation a break occurs at 2. At 3 two more movements occur at *E*, followed by single movements at *D* and *C*. In the movements starting at 4, each movement at *E* is followed by one at *D* and at *C*.

Room temperature 22°C. The vertical lines are 5 seconds apart.

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[6] Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 215.

[7] We must consider the possibility of a conflict of pacemakers but there is no evidence of such a conflict in the present case.

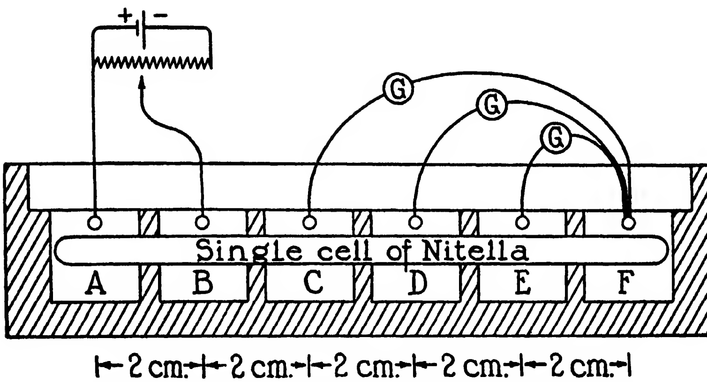

FIG. 2. Diagram of a series of paraffin cups *A* to *F*, with a single cell of *Nitella* passing through all of them. *GGG* represent string galvanometers (three strings inserted in the single magnetic field of a Type A Cambridge string galvanometer) with vacuum tube amplifiers, arranged as short period voltmeters. Ag-AgCl electrodes dip into the cups. Cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87. *A*, *B*, *C*, *D*, and *E* are in contact with 0.01 M NaCl. *F* is in contact with 0.01 M KCl. The cell had been kept for 2 hours in 0.01 M NaCl before use.

SUMMARY

A series of negative variations passing along the cell may reach a region where only every other variation registers. This condition may be temporary. It would seem to depend on a local change in the refractory period.

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CHEMICAL RESTORATION IN NITELLA

II. RESTORATIVE ACTION OF BLOOD

BY W. J. V. OSTERHOUT

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(Accepted for publication, July 9, 1935)

The irritability[1,2] of *Nitella* depends on a substance (or a group of substances) which can be dissolved out of the protoplasmic surface by distilled water. From the water in contact with the cells substances can be extracted by organic solvents and these substances when redissolved and applied to the cell restore the irritability. The experiments indicate that these substances are organic in nature.[3]

If this applies to irritability in general such substances must occur in animals. It is therefore of considerable interest to find that irritability and the potassium effect[1] can be quickly restored by blood.

Cells which had lost their irritability were placed in blood plasma. After 15 seconds many were found to be irritable when tested in the usual manner by applying an electrical stimulus (300 mv. D.C.). In some cases a longer treatment[4] (up to 2 minutes) was required.

In general the forms of the action current were like those found in control cells although in many cases they belonged to types which, though occurring in normal cells, are not those most commonly encountered in such cells.

Since it has been found[1] that calcium can restore irritability it was necessary to exclude its action. It was accordingly removed at the start by adding sodium oxalate (1 gm. to each liter of freshly drawn blood) after which the blood plasma was diluted with 4 parts of distilled water. As the inorganic substances remaining in the blood

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 987.

[2] By this is meant the ability to give action currents as the result of electrical stimulation.

[3] Osterhout, W. J. V., and Hill, S. E., *Proc. Soc. Exp. Biol. and Med.*, 1934–35, **32**, 715; also S. E. Hill, unpublished results.

[4] A much longer exposure resulted in some cases in a falling off of irritability.

after the removal of calcium have been found to be ineffective in restoring irritability and the potassium effect we may conclude that the active agents are organic substances.[5]

The experiments were performed on *Nitella flexilis* Ag. at temperatures of from 20–22°C. The technique employed was that described in a previous paper;[1] the action current and the responses to KCl were recorded photographically. The cells were kept in distilled water until they no longer gave action currents when stimulated in the usual way with 100–400 mv. D.C. This usually took 2 days. After treatment with blood they were tested for irritability by the same electrical stimulus.

The blood was drawn into vessels containing oxalate, placed in the centrifuge to remove blood corpuscles, and the plasma was diluted for use.

There was some irregularity in the results, *e.g.* in some cases the first response was incomplete (*i.e.* the loss of P.D. amounted to from 10 to 50 per cent, instead of being complete as in the typical action current[6]). In such cases a second or third stimulus sometimes produced a complete response. In a few cases three successive stimuli failed to elicit any response but it is possible that these cells were in poor condition.

Human blood and that of the sheep, calf, and cat[7] gave essentially the same results.

What is said of irritability applies in general to the potassium effect. Since the latter depends on the outer protoplasmic surface and the former more especially on the inner surface[6] we might expect the potassium effect to be restored more promptly than irritability by the action of substances added to the external solution. This seems to be true in general as has been stated[1] in describing experiments with NH_3, but in the case of blood there are many exceptions. This is perhaps less surprising in view of the fact that the restorative substances in blood appear to penetrate with great rapidity.[8] The fact that these sub-

[5] This is confirmed by extraction experiments by S. E. Hill (unpublished results).

[6] Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 215.

[7] In some cases whole blood diluted with 6 parts of distilled water to produce hemolysis (without oxalate) was employed with essentially the same results.

[8] When it is stated that irritability was restored after cells had been 15 seconds in blood plasma it must be remembered that after they had been removed and placed in contact with 0.01 M NaCl the restorative substances continued to diffuse inward (as well as outward). But in such cases the restoration was complete in less than a minute after the cells first came in contact with the blood plasma.

stances are organic may help to explain their rapid penetration (it may be noted that chloroform is an example of an organic substance which penetrates with extreme rapidity[9]).

When an action current preceded the restoration of the potassium effect the latter was not regarded as due to the application of blood since the action current can itself cause the restoration of the potassium effect.[10]

When a brief treatment with blood had failed to restore irritability replacement of 0.01 M NaCl by 0.01 M KCl gave the potassium effect in many cases thus showing that the restoration of the potassium effect by blood can be more rapid than the restoration of irritability.

The substances which are responsible for the potassium effect and for irritability appear to be of widespread occurrence. The fact that they are found in animals suggests that they may be important in connection with the irritability of muscle and nerve and it seems possible that they may be concerned in various disturbances of nervous function. In that case it would be interesting to know how early they appear in animal ontogeny and phylogeny.

SUMMARY

Cells of *Nitella* exposed to distilled water lose their ability to produce action currents and to distinguish electrically between sodium and potassium. This ability was quickly restored by exposure to blood plasma deprived of calcium. Human blood and that of the cat, calf, and sheep gave essentially the same results.

The active agents appear to be organic substances.

[9] *Cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 673.

[10] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1934–35, **18**, 681.

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AN ATTEMPT TO FORMULATE A QUANTITATIVE THEORY OF MEMBRANE PERMEABILITY

BY TORSTEN TEORELL*

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The fundamental theories of electrolyte diffusion (Nernst,[1] Planck[2]) take into account the electrostatic forces that coöperate with the "osmotic" forces to cause the migration of ions. In these theories, however, no assumption has to be made as to the origin of the charge on the particles in the solution. Accordingly, we may expect that their predictions regarding ionic diffusion may be extended to include other cases of diffusion, where other charged elements are present, regardless of the constitution, shape, etc., of these elements. Diffusion of an electrolyte through a membrane may be such a case. The membrane may be regarded as having a charge due either to "adsorption," "dissociation," or "polar character," etc., but it is not necessary to make any further assumptions as to its nature. The effect of the membrane is regarded as that of an "added ion."

In order to demonstrate the usefulness of treating permeability problems as cases of simple diffusion we shall—for the sake of clarity, in a highly simplified way—try to show that the so-called "concentration effect" when NaCl diffuses across a "negative" membrane is theoretically predictable:

	Membrane		
	X—	X—	
NaCl	Cl_1	Cl_2	NaCl
a_1	Na_1	Na_2	a_2
1	1′	2′	2

From one side of the membrane, NaCl, having the activity a_1,

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[1] Nernst, W., *Z. physiol. Chem.*, 1888, **2**, 617; 1889, **4**, 154.

[2] Planck, M., *Wied. Ann.*, 1890, **40**, 561.

diffuses to the other side where the activity is a_2. The membrane may be represented as consisting of negative, immobile ions of the activity X, which is assumed to be constant throughout the membrane. In the steady state the ionic activities in the membrane surface layers may be Na_1, Cl_1 and Na_2, Cl_2. For electroneutrality it may be assumed that $Na_1 = (Cl_1 + X)$ and $Na_2 = (Cl_2 + X)$. Although the concentration of Na in the membrane differs from that of Cl the flux will be equal because the forces ("osmotic" plus electrical) operating on them are not equal. If the diffusion from 1′ to 2′ is sufficiently

TABLE I

X or membrane "activity" = 1. Membrane negative. Mobility relation $u:v$ in the membrane the same as in water. (Signs refer to the dilute solution in the external circuit.)

a_1	a_2	Partial E.M.F		Total E.M.F. mv.
		Boundary mv.	Diffusion mv.	
100	10	+1.1	−13.2	−12.1
10	1	+10.9	−12.1	−1.3
5	0.5	+20.5	−12.0	+8.5
1	0.1	+46.2	−5.4	+41.8

slow, the ionic distribution across 1-1′ and 2-2′ respectively approaches a thermodynamic equilibrium and we may write as an approximation

$$a_1^2 = Na_1 \cdot Cl_1 = Na_1 \cdot (Na_1 - X) \tag{1}$$

and

$$a_2^2 = Na_2 \cdot (Na_2 - X) \tag{2}$$

Evidently there are 2 "boundary" potentials present here between 1-1′ and 2-2′, which sum up to

$$\text{Total boundary potentials} = 58 \log [(a_1 \cdot Na_2) + (a_2 \cdot Na_1)] \tag{3}$$

Besides these we have a "diffusion" potential between 1′-2′. As X is constant along the distance in the diffusion layer, the "concentration" gradient of Cl also is linear, because, as shown by Planck, the *total* concentration (Na) must always fall off linearly. Under such condi-

tions the 1'-2' potential can be most simply expressed by Henderson's formula,[3] which here reduces to

$$\text{Diffusion potential} = [(u - v) \div (u + v)] \cdot$$
$$58 \log [\text{Na}_1(u + v) - X \cdot v] \div [\text{Na}_2(u + v) - X \cdot v] \quad (4)$$

u and v are the constant "mobilities" (inverse friction coefficients) of Na and Cl. The total E.M.F. of the membrane is the sum of equations 3 and 4:

$$\text{Total E.M.F.} = 58 \left[\log \frac{a_1 \cdot \text{Na}_2}{a_2 \cdot \text{Na}_1} + \frac{u - v}{u + v} \log \frac{\text{Na}_1\,(u + v) - X \cdot v}{\text{Na}_2\,(u + v) - X \cdot v} \right] \quad (5)$$

For calculations, Na_1 and Na_2 have to be expressed in terms of a_1 or a_2 and X, which is possible by means of equations 1 and 2.

If $X > 0$, it is found that the total E.M.F. is bound to vary with the absolute activities a_1 and a_2, even if the ratio $a_1 \div a_2$ is kept constant. This circumstance, however, is just what has been experimentally observed in a great many cases and has been called "concentration effect" (*cf.* Beutner,[4] Michaelis,[5] Osterhout[6]). Table I shows the numerical results of calculations according to equation 5 for this particular case.

Using NaCl the sign of the total E.M.F. will depend upon the relation between X and the concentrations of the external solutions. In the comparable experiments of Beutner and of Michaelis, *et al.*, this relation seems to be such that only positive E.M.F. values are observed. These values, however, increase with decreasing concentration, as predicted by the present theory, and the limit + 58 mv. is approached according to both experiments and calculations.

Further discussions of this and other permeability problems, regarded as cases of "forced" diffusion, will, it is hoped, be presented in other communications.

[3] Henderson, P., *Z. physik. Chem.*, 1907, **59**, 118.

[4] Beutner, R., Physical Chemistry of Living Tissues and Life Processes, Baltimore, Williams and Wilkins Co., 1933.

[5] Michaelis, L., *Kolloid-Z.*, 1933, **62**, 1. Michaelis, L., Ellsworth, R. McL., and Weech, A. A., *J. Gen. Physiol.*, 1926–27, **10**, 671.

[6] Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, **12**, 761.

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THE KINETICS OF PENETRATION

XII. HYDROGEN SULFIDE

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(Accepted for publication, July 2, 1935)

INTRODUCTION

It has been shown in former papers[1,2] that the penetration of the weak base ammonia and the strong base guanidine into the large multinucleate cells of *Valonia macrophysa* Kütz is probably preceded by a reversible chemical reaction between the base and one or more acidic constituents of the protoplasm. The question now arises whether the entrance of weak and strong acids also depends on analogous reactions with basic constituents of the protoplasm.

As in the case of bases it seemed possible to test this by determining the rate of entrance of the acid at the early part of the process when the internal concentration is still small as compared with the external concentration.

As a representative of the weak acids hydrogen sulfide was selected.

EXPERIMENTAL

The rate of entrance was studied in two ways. In one series of experiments the concentration of the sulfide ion was kept constant and the external pH varied. In the other the pH was kept constant and the concentration of sulfide ion varied.

The concentration of hydrogen sulfide in water, according to some recent work by Wright and Maass,[3] at 25°C. and a partial pressure of 755 mm., is 0.1010. Since sea water has an ionic strength of about 0.7 some slight salting out is to be expected. Nevertheless the solubility of the gas in sea water is sufficient to permit the use in closed systems of H_2S concentrations fairly high from the biological viewpoint, without the development of dangerous pressures or the loss of

[1] Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1935, **21**, 125.

[2] Jacques, A. G., *Proc. Nat. Acad. Sc.*, 1935, **21**, 488.

[3] Wright, R. H., and Maass, O., *Canad. J. Research*, 1932, **6**, 94.

too much gas. In order to reduce the latter as much as possible and to keep it uniform, only enough sulfide-containing sea water for a single determination was made up at one time. This was mixed with the least possible agitation in a closed vessel and transferred at once to a bottle which it filled completely. The cells for experiment were added at this point and the bottle stoppered at once without gas space above the liquid. To produce uniform results it was found necessary to use cells of fairly uniform size and to stir. The stirring apparatus described in a previous paper[2] was used.

The pH was not determined in the sea water sample to which the cells were to be exposed as this would have involved delay and loss of gas. Instead, for each determination parallel samples were prepared, one for pH and the other for cell exposure. The pH was determined colorimetrically, using the Hellige double wedge colorimeter, the reading being referred to calibration curves as described in a previous paper.[4] After the exposure of the cells, the pH of the sea water was again determined as a check on the loss of gas. If it had changed by more than 0.15 pH unit, the run was rejected. This seldom happened.

At the outset it was our intention to use the pH determination to calculate the concentration of molecular hydrogen sulfide in the sea water from the known total sulfide concentration. However, difficulties arose which reflected seriously on the validity of these calculations and it was found that the object of the work could be attained without knowing the true pH.

After exposure the cells were washed rapidly in a stream of distilled water and dried with filter paper. The sap was then extracted by the following technique which reduces gas loss from the sap to a minimum.

Using a 1 ml. "tuberculin" syringe with a fine needle, the cell was punctured and the sap was forced into the syringe, by squeezing the cell, against the opposing pressure furnished by the friction of the piston against the barrel. The sap of the first cell carried air from the needle into the syringe and this was expelled together with the sap by reversing the syringe and returning the piston to the "empty" position. This left the syringe empty but with the needle full of sap, possibly slightly deficient in H_2S, and sealed by a thin layer of liquid between the piston and barrel. Any further sap forced into the syringe was thus forced into a closed space without gas space. The sample (usually 1 ml.) was measured directly in the syringe and at once introduced beneath the surface of a known quantity of a standard iodine solution. The excess of iodine was then determined by reducing it with standard thiosulfate solution in the presence of a starch indicator. Thus the amount of iodine used to oxidize the sulfide was known and from it the sulfide concentration of the sap was calculated.

As always, the question of injury remains to be considered. Observations by the eye and by feeling suggested that no injury had occurred where the exposure was less than 15 minutes. Moreover the cells not extracted, after return

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[4] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1931–32, **15**, 537.

to running sulfide-free sea water, showed no greater mortality rate than control cells not exposed to sulfide. When the exposure was for 2 hours, as in the determination of equilibrium values, the unused cells exposed to the highest H_2S concentration after return to running sea water showed a slightly lower survival rate. But those which did survive appeared to be uninjured and had no more sulfate in the sap than the controls.

EXPERIMENTAL RESULTS

The rates of entrance of sulfide at the same total external sulfide concentration but at different pH (hereafter called Series 1) are given

TABLE I

Penetration into Valonia of H_2S from Sea Water at Various pH and Constant Total Sulfide Concentration

Series 1

Apparent pH...	8.20	8.02	7.70	7.30	7.04	Unknown*	Unknown*
Equivalent concentration at equilibrium..	0.0062	0.0122	0.0163	0.0246	0.0322	0.0475	0.0568
Time	Equivalent concentration of sulfide in the sap						
min.							
0.5		0.00118			0.00246		
1	0.00056	0.00163	0.00231	0.00313	0.00403	0.00623	0.00690
2	0.00128	0.00232	0.00387	0.00502	0.00690	0.00934	0.0126
3	0.00172	0.00336	0.00465	0.00715	0.00890	0.0131	0.0175
4	0.00195	0.00372	0.00585	0.00920	0.0106	0.0159	0.0196
5	0.00235	0.00442	0 00672	0.0112	0.0135	0.0188	0.0220
7	0.00272	0.00548	0.00785	0.0139	0.0177	0.0227	
10	0.00368	0.00368	0.00919	0.0164			

* Solutions slightly milky so that pH could not be determined.

in Table I and Fig. 1. In this case, as in all the other figures, the curves are drawn free-hand to give an approximate fit. Where possible in the table, the "apparent" pH is given, but this, as indicated above, is probably not significant. The "equilibrium concentration" is much more important, since, as will be shown later, it is probably a fairly accurate measure of the external concentration of molecular hydrogen sulfide.

The rates of entrance where the external concentration of sulfide was varied (hereafter called Series 2) and the external pH kept constant are given in Table II and Fig. 2.

The method by which the pH was adjusted requires some comment. Throughout the experiment the same solution of 0.6 N sodium sulfide was used, and the same 0.6 N solution of hydrochloric acid was employed to adjust the pH, and

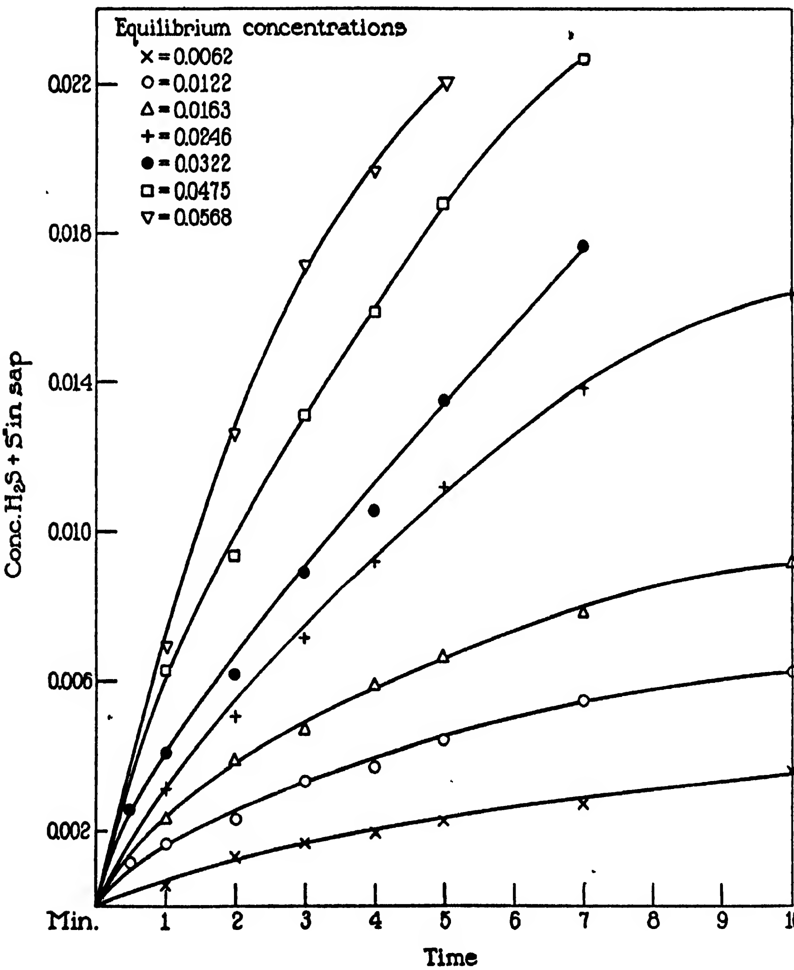

FIG. 1. Penetration of H_2S into *Valonia* from sea water containing varying concentrations of undissociated H_2S, brought about by keeping the total H_2S constant and varying the pH (Series 1).

these were added to the sea water in the same proportions. As is now generally believed, the buffer effect of normal sea water depends upon the presence of 0.002 N $NaHCO_3$ and the dissolved CO_2 which is in equilibrium with the CO_2 of

the atmosphere. It is not surprising, therefore, that its buffer capacity is low. In some recent measurements on sea water collected near Newport Mitchell and Rakestraw[5] found that by the addition of 0.75 cc. of 0.075 N HCl solution to 100 ml. of sea water the pH was lowered from 8.2 to 7.0 The amount of acid corresponds to about 0.09 cc. of 0.6 N HCl^-. The smallest amount of 0.6 N HCl used by us per 100 ml. of sea water was 2.85 ml. which was the amount required to bring the pH of a 100 ml. sample of sea water 0.02 N with respect to sulfide between 7.0 to 7.1. Clearly therefore even in this case the amount of the acid used up by the ordinary buffer system of the sea water is only a fraction of that required by the new sulfide system added. Over the range of sulfide concentrations studied by us it is the added sulfide system which controls the pH and hence as long as the ratio of total sulfide to added free acid remains unchanged the pH

TABLE II

Penetration into Valonia of H_2S from Sea Water at Various Total Sulfide Concentrations and Constant pH

Series 2

Equivalent concentration in sea water Total $S^=$...........	0.0200	0.0500	0.0750	0.100	0.150	0 200
Time	Equivalent concentration of sulfide in the sap					
min.						
1	0.00090	0.00216	0.00320	0.00403	0.00617	0.00851
2	0.00143	0.00350	0.00537	0.00698	0.0106	0.0149
3	0.00186	0.00467	0.00746	0.00890	0.0136	0.0182
4	0.00226	0.00548	0.00883	0.0106	0.0161	0.0228
5	0.00258	0.00677	0.0115	0.0135	0.0210	0.0282
Equilibrium concentration......	0.00625	0.0160	0.0250	0.0322	0.0468	0.0640

should remain constant. The apparent pH did indeed remain approximately constant, varying from 7.0 to 7.1. It should be noted that at pH 7.0 the second dissociation of H_2S is negligible since pK_2 = about 15.

The uncertainty with respect to the proportion of molecular hydrogen sulfide to ionized sulfide is not quite so great in the case of the sap as in the sea water. In the first place, apparently no polysulfide (which gives the solution a faint yellow color) finds its way into the sap and no precipitate of sulfur occurs there even when the total sulfide concentration is comparatively high and the pH is far below 6.5. The uncertainty with respect to the dissociation constant persists, but by analogy with H_2CO_3 it is permissible to guess the extent of the change of pK_1 as the ionic strength increases from 0 to 0.61. According to D. A. MacInnes

[5] Mitchell, P. H., and Rakestraw, N. W., *Biol. Bull.*, 1933, **64**, 437.

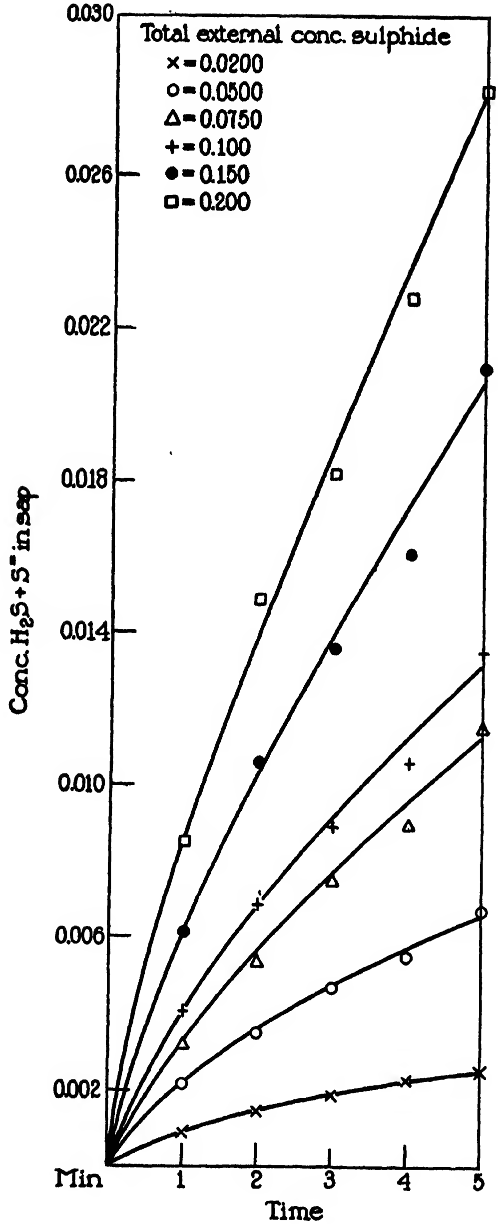

FIG. 2. Penetration of H_2S into *Valonia* from sea water containing varying concentrations of undissociated H_2S, brought about by keeping the pH constant (7.0−7.1) and varying the total sulfide concentration (Series 2).

and T. Shedlovsky[6] the limiting value for the first dissociation constant of carbonic acid, pK_1, may be taken as 6.395 at 20°C. They find that this value fits in well with the recent values determined by Güntelberg and Schiödt[7] for pK'_1 at various concentrations of KCl. By interpolating from the curve of Güntelberg and Schiödt we obtain for pK'_1 at 0.61 N KCl, 6.020, a shift of −0.375 unit.[8]

The pK_1 value of H_2S is not well known. According to Walker and Cormack[9] at 18°C. in 0.008 molar solution (ionic strength 0.032) pK'_1 is 7.245; and according to Auerbach[10] under nearly the same conditions, 7.039. Since these are in relatively dilute solutions they may be provisionally taken as the limiting values at zero ionic strength. For present purposes the average 7.142 is taken. pK'_1 at 0.61 ionic strength in KCl, which approximates that of the sap, is now obtained by assuming that the correction applied to H_2CO_3 may be applied here, whence $pK'_1 = 7.142 - 0.375 = 6.767$.

Using this value rounded to 6.77 and the pH determination in the sap, Table III has been obtained, which shows the pH change in sap on exposure of the cells to sulfide sea water for the most dilute and most concentrated solutions in Series 2.

These results indicate that as H_2S penetrated the cell the pH in the sap decreased very rapidly to the point where the sulfide was practically all present as undissociated hydrogen sulfide.

DISCUSSION OF RESULTS

In the case of ammonia it is possible to calculate the concentration of molecular NH_3 in the sea water from the total concentration and the pH. In the present case, as pointed out above, owing to the uncertainty of the pH measurements this cannot be done. Fortunately, however, we can determine these values directly, since it seems clear that they are identical with the equilibrium concentrations.

In a former paper, Osterhout[11] has shown that at equilibrium[12] the

[6] Private communication.

[7] Güntelberg, E., and Schiödt, E., *Z. physik. Chem.*, 1928, **135**, 393.

[8] It is worth while noting that if the value for pK_1 given by Kendall, which has hitherto been regarded as correct, *viz.* 6.493, is taken the correction to ionic strength of 0.71 (that of sea water) would be about 0.48 unit. This is in good agreement with the findings of Buch and his coworkers (Buch, K., Harvey, H. W., and Wattenberg, H., *Naturwissenschaften*, 1931, **19**, 773) that the decrease in the pK'_1 value of H_2CO_3 in sea water is about 0.5 pH unit, pK_1 being taken as 6.472.

[9] Walker, J., and Cormack, W., *J. Chem. Soc.*, 1900, **77**, 5.

[10] Auerbach, F., *Z. physik. Chem.*, 1904, **49**, 217.

[11] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925, **8**, 131.

[12] Osterhout found that it required about 1 hour to establish equilibrium, and his measurements were made after 2 hours. Our cells behaved similarly and we also allowed them to run 2 hours.

concentration of molecular hydrogen sulfide in the sea water is equal to the total sulfide in the sap. But, as Table III indicates, the rate of fall of pH in the sap was such that at the end of 5 minutes, even in the most dilute solution studied, it had fallen to the point where practically all the sulfide must have been present in the undissociated form. We may modify the above statement therefore to read that at equilibrium the concentration of molecular hydrogen sulfide in the sea water is equal to the concentration of molecular hydrogen sulfide in the sap. This means that from the lowest to the highest concentrations of total sulfide in the sea water studied, the equilibrium concentration is a true measure of the concentration of molecular hydrogen sulfide in the sea water.[13]

TABLE III

Change in the pH of the Sap of Valonia with Entrance of H_2S

External sulfide concentration.........	0.02 N		0.2 N	
Time	pH of sap	Molecular H_2S in sap	pH of sap	Molecular H_2S in sap
min.		*per cent*		*per cent*
0	6.06	84	6.10	83
½	5.77	91		
1	5.71	92	5.34	96
3	5.42	96		
5	5.30	97	5.04	98

In Fig. 3 we have plotted the results of Series 1 to show the increase in the total sulfide of the sap in 1, 2, 3, 4, and 5 minutes with the

[13] It is interesting to consider what would happen if the association of sulfide to molecular H_2S in the sap were not substantially complete over the range studied by us. The equilibrium concentration which is actually an analytical determination of the *total* sulfide in the sap would no longer be a true measure of the external concentration of molecular H_2S at every concentration. It would in fact be too high at the lower concentrations, the errors gradually becoming less as the sulfide content outside increased until a concentration would be reached where the two values would be identical. Any plot of rate as ordinates against uncorrected equilibrium concentrations would necessarily have too little concave curvature. And if the "equilibrium values" were corrected to give concentrations of free H_2S, the curve would be concave to the x-axis. Fortunately such conditions do not apply in the case of H_2S.

increase in the equilibrium concentration which is, as we have pointed out above, a measure of the molecular hydrogen sulfide in the sea water. Provisionally the ordinates which are concentrations may be considered as "rates," in the sense that they give the amount of sulfide

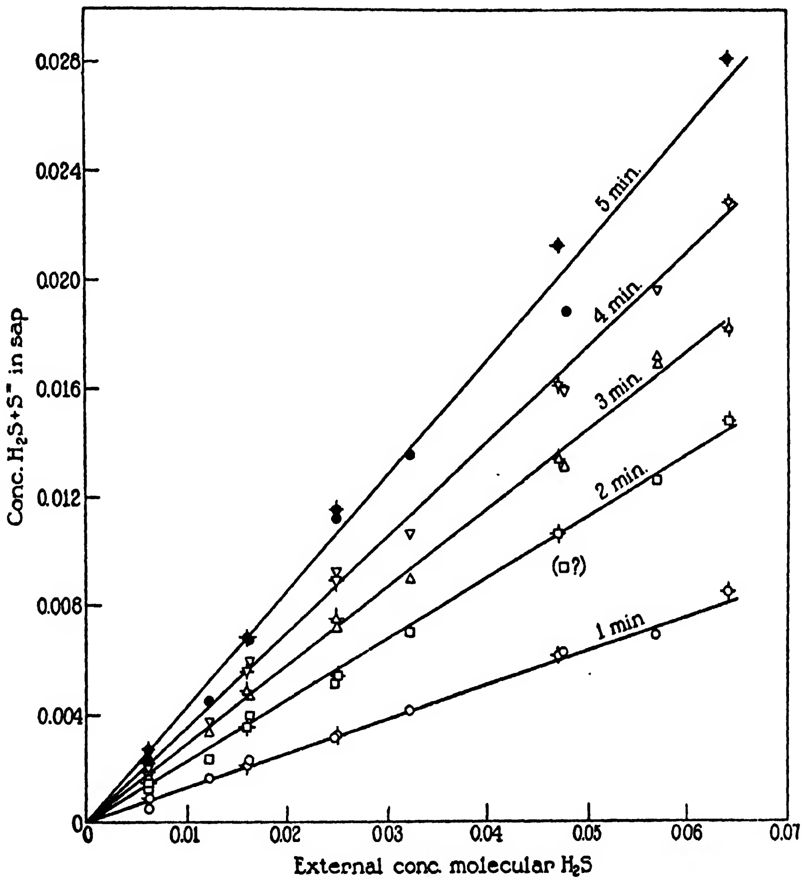

FIG. 3. Rates of entrance of H_2S into *Valonia* at periods from 1 to 5 minutes at various external concentrations of undissociated H_2S. Plain symbols refer to Series 1 and crossed symbols to Series 2.

entering the sap when the time units are 1 to 5 minutes. The momentary rates, $\frac{d(H_2S)}{dt}$, cannot be obtained easily since the form of the rate curves is not known.

It is seen that the "rate of increase" of sulfide in the sap is directly proportional to the concentration of molecular H_2S in the sea water over the range from 1 to 5 minutes, since the curves of Fig. 3 are clearly linear and pass through the origin. This behavior differs from that of ammonia or guanidine where the rate is not a linear function of the concentration of free ammonia or guanidine in the sea water.[1]

In the case of ammonia, as Osterhout has shown, the form of the rate curve, x against $(NH_3)_o$, can be derived from the relationship $(NH_3)_o(HX_b - NH_4X_e) = K(NH_4X_e)$, which is the expression for the equilibrium of the reversible reaction $NH_3 + HX \rightleftharpoons NH_4X$. The subscripts o, b, and e refer respectively to the outside solution, and the beginning and the end of the reversible reaction, and x is the total ammonia concentration in the sap; *i.e.*, ionized and unionized.

The equation gives only the relationship between the concentration of NH_4X_e and $(NH_3)_o$. But since the rate x is assumed to be proportional to NH_4X_e we can substitute for NH_4X_e, k_1x in the equation, thus

$$(NH_3)_o(HX_b - k_1x) = kk_1x$$

Putting

$$kk_1 = k_2, \qquad x = \frac{(NH_3)_o HX_b}{k_2 + k_1(NH_3)_o}$$

$$\frac{\partial x}{\partial (NH_3)_o} = \frac{k_2 HX_b}{k_2 + k_1(NH_3)_o} \; *$$

* k and HX_b may be regarded as constants for any one collection of cells of the same size since these will have roughly the same permeability, and the same concentration of HX in the protoplasmic surfaces. But for different collections made at different times and kept under different conditions they may be quite different.

In the case of H_2S, however, $x = k'a$ by experiment, where a is the external concentration of molecular H_2S and x is the value of $H_2S + S^{=}$ in the sap, and hence $\left(\frac{\partial x}{\partial a}\right)_t = k'$, where t signifies time. Superficially therefore it would seem that the modes of entrance for ammonia and H_2S are different.

But the question may be raised whether the H_2S curves really are linear. It might be supposed, for example, that if the external con-

centration of molecular H_2S were calculated from the pH a different set of curves would be obtained. Actually this turns out to be the case if we use the apparent pH values (Series 1) to calculate the concentration of molecular H_2S. In this case curves concave to the x-axis are obtained. However, the pH values are so unreliable that no results calculated from them can be regarded as trustworthy. Fortunately the linear relationship for the H_2S curves is supported by the data of Series 2 where it is unnecessary to know the equilibrium concentration, since the sulfide sea waters were prepared in such a way as to make the pH constant while the total sulfide concentration was varied. Under these conditions the concentration of molecular hydrogen sulfide in the external solution must be directly proportional to the total sulfide concentration, so that if we plot the rates against the total sulfide concentration it would give the same form of curve, though with a different slope as the plot of rates against molecular H_2S. Such a plot is given in Fig. 4. As before, there is no question that the five curves obtained are linear.

It may be noted that the apparent pH in this series varied from 7.0 to 7.1. This must be regarded as reasonably constant for this type of experiment, but in reality it is a fairly large deviation. It is not surprising therefore that some of the points of the plot do not lie closely on the curves. But the absence of a systematic drift from the linear relationship is perfectly clear. As a further test of the linear curves of Series 1 (Fig. 3) the equilibrium concentrations for Series 2 were also obtained and the rates against these values have been plotted in Fig. 3 where they are distinguished as crossed symbols, thus ⧊, ⌖, etc. It will be observed that they fit smoothly onto the curves derived from Series 1.

An interesting deduction from a comparison of the two series leads to the conclusion that ionic entry plays no part in the process.

Comparing the second column of Table I with the second column of Table II we find that the rates and equilibrium concentrations were about equal. Yet in the first case the total sulfide was 0.1 N and the ionic sulfide therefore 0.1 N − 0.0062 N = 0.0938 N, while in the second case since the total sulfide was 0.02 N the ionic sulfide was 0.02 N − 0.00625 N = 0.01375 N. The rate is therefore unaffected by an almost sevenfold increase in the concentration of ionic sulfide.

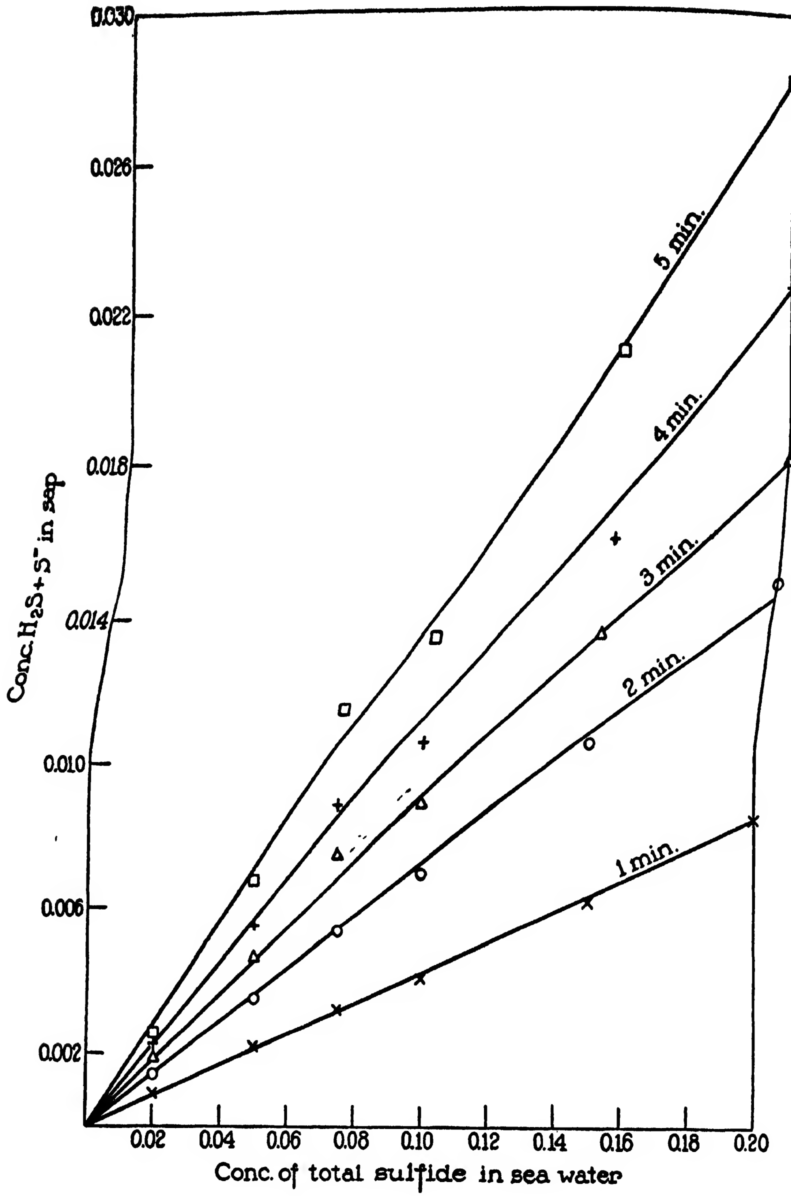

FIG. 4. Rates of entrance of H_2S into *Valonia* at periods from 1 to 5 minutes at various external total sulfide ($H_2S + S^=$) concentrations. pH constant (7.0 – 7.1).

The fact that H_2S curves are clearly linear and the ammonia curve clearly not, suggests that the mode of entrance is different.

However, the two cases are not strictly analogous, because in the ammonia case, at the pH of the sap, a large proportion of the ammonia that enters is transformed to ammonium ion. It is true that the entrance of ammonia raises the pH. According to the results of Cooper and Osterhout,[14] during the first few minutes of penetration from sea water 0.005 N with respect to NH_4Cl, it may rise from the normal value of 5.8–6.0 to 7.2. For ammonia, according to Noyes, Kato, and Sosman[15] pK_b at 25°C. is 4.72. To get pK'_b in the sap (a solution of 0.61 ionic strength the chief salt of which is KCl) we assume the same correction as was applied in the case of pK_1 of H_2CO_3, *viz.* 0.38. pK'_b is therefore $4.72 - 0.38 = 4.34$. Since at 25° C. the limiting value of $K_w = 14.00$, $pK'_{ab} = 9.66$.[16] At pH = 7.2 therefore $\alpha = 99.7$ per cent and the undissociated fraction is negligible. Hence the back diffusion due to the formation of NH_4X at the interface between the sap and the protoplasm must be very small.

In the case of H_2S, as Table III shows, the pH of the sap falls off so rapidly that practically all the sulfide is present in the unionized form, hence the effect of this back pressure on $\left(\frac{\partial x}{\partial t}\right)_{(H_2S)_o}$ and on $\left(\frac{\partial x}{\partial (H_2S)_o}\right)_t$ must be taken into account.

Let us consider the general case of the penetration of a weak acid or base. Let us assume that only molecules penetrate the non-aqueous surface layer of the protoplasm where they react to form a salt which passes through this layer and that in consequence the rate of passage through the protoplasm is proportional to the concentration of this salt. For the rate we can write

$$\frac{dx}{dt} = k'(y_o - y_i)$$

where x is the total acid or base in the sap (ionized and unionized), y is the concentration of the salt formed at the interfaces, and o and i refer to the external and internal interfaces respectively.

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[14] Cooper, W. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 117.

[15] Noyes, A. A., Kato, Y., and Sosman, R. B., *Z. physik. Chem.*, 1910, **73**, 1.

[16] Note that 14.00 is the value $K_w = (OH)(H)$, *i.e.* that it is in terms of activities. The value here used is a recent one by Harned and Hawes (Harned, H. S., and Hawes, W. J., *J. Am. Chem. Soc.*, 1933, **55**, 2194).

According to the principles which Osterhout has applied to the ammonia case, for the layer of the protoplasm in contact with the external solution

$$a(c_o - y_o) = k_3 y_o$$

and for the layer of protoplasm in contact with the sap

$$(1 - \alpha)x(c_i - y_i) = k_3 y_i)$$

where c is the concentration of base or acid in the protoplasm which reacts with the acid or base the entrance of which is being studied, α is the degree of dissociation, and a is the concentration of undissociated molecules of the penetrating acid or base which determines the reaction in the surface layer of the protoplasm. Hence

$$y_o = \frac{ac_o}{k_3 + a_o}$$

and

$$y_i = \frac{(1 - \alpha)x\, c_i}{k_3 + (1 - \alpha)x}$$

and

$$\frac{dx}{dt} = K\left(\frac{ac_o}{k_3 + a} - \frac{(1 - \alpha)x\, c_i}{k_3 + (1 - \alpha)x}\right)$$

For the case of ammonia the last term drops out because $1 - \alpha$ is zero or practically so.

For other cases under the conditions that $(1 - \alpha)$, c_o and c_i[17] remain constant the above expression can be integrated for a constant value of a to give the relationship between t and x,[18]

---

[17] In the case of H_2S, for example, $(1 - \alpha)$ becomes almost at once nearly unity because of the decrease in the internal pH. c_o and c_i may also be regarded as constant and equal to each other in the thin unstirred layers in which the reaction occurs.

[18] The steps in integration are as follows. Collect constants, to get

$$\frac{dx}{dt} = \left(A - \frac{BX}{1 + CX}\right)$$

in which

$$A = \frac{Kac_o}{k_3 + a}, \qquad B = \frac{K(1 - \alpha)c_i}{k_3}, \qquad C = \frac{(1 - \alpha)}{k_3}$$

Then

$$\frac{dx}{dt} = \frac{A + ACX - BX}{1 + CX} = \frac{A + B'X}{1 + CX}$$

(Footnote continued on following page)

$$t = \frac{1}{C\left(\frac{K_{e_0a}}{k_3+a}\right) - B} \log\left[\frac{K_{e_0a}}{k_3+a} + \left(\frac{CK_{e_0a}}{k_3+a}\right) - B\right]x$$

$$+ \frac{C}{\left(C\frac{K_{e_0a}}{k_3+a} - B\right)^2}\left[\frac{K_{e_0a}}{k_3+a} + \left(C\frac{K_{e_0a}}{k_3+a} - B\right)x\right]$$

$$- \frac{K_{e_0a}}{k_3+a}\log\left[\frac{K_{e_0a}}{k_3+a} + \left(\frac{CK_{e_0a}}{k_3+a} - B\right)x\right]$$

$$- \frac{1}{C\left(\frac{K_{e_0a}}{k_3+a} - B\right)}\log A - \frac{C}{\left(C\frac{K_{e_0a}}{k_3+a} - B\right)^2}\left[\frac{K_{e_0a}}{k_3+a}\right.$$

$$\left. - \frac{K_{e_0a}}{k_3+a}\log\frac{K_{e_0a}}{k_3+a}\right]$$

(Footnote 18 continued from preceding page)

where $AC - B = B'$, or

$$\frac{dt}{dx} = \frac{1+CX}{A+B'X}, \qquad \frac{dx}{A+B'X} + \frac{cxdx}{A+B'X}$$

$$\int dt = \int\frac{dx}{A+B'X} + C\int\frac{Xdx}{A+B'X} + I$$

or

$$t = \frac{1}{B'}\log(A+B'X) + \frac{C}{(B')^2}[A+B'X - A\log(A+B'X)] + I$$

when $t = 0$, $x = 0$, hence $I = -\frac{1}{B'}\log A - \frac{CA}{(B')^2}(A - \log A)$ when

$$t = \frac{1}{B'}\log(A+B')X + \frac{C}{(B')^2}[A+B'X - A\log(A+B'X)]$$

$$-\frac{1}{B'}\log A - \frac{C}{(B')^2}(A - A\log A)$$

Substituting in the expression for B' we get

$$t = \frac{1}{AC-B}\log[A+(AC-B)X] + \frac{C}{(AC-B)^2}[A+(AC-B)X -$$

$$A\log(A+(AC-B)X)] - \frac{C}{AC-B}\log A - \frac{C}{(AC-B)^2}(A - A\log A)$$

Substituting for A, which contains a, we get the expression above.

In this expression $C = \frac{(1 - \alpha)}{k_3}$ and $B = \frac{K(1 - \alpha)c_1}{k_3}$.[19]

This shows that if a (p. 284) is the external concentration of molecular H_2S and x is the value of $H_2S + S^=$ inside, the value of $\left(\frac{\partial x}{\partial a}\right)_t$ is not constant when the method of entrance involves reversible chemical reactions between H_2S and a constituent of the protoplasm. Suppose, however, the acid or base enters by diffusing in the non-aqueous layers of the protoplasm in undissociated form. We can apply to this case the ideas already developed by Osterhout[20] and by Longsworth[21] for cell models with the guaiacol-p-cresol mixture as the non-aqueous phase.

Then we can write

$$\frac{dx}{dt} \sim (y_o - y_i)$$

where y is the concentration of the diffusing molecules in the thin unstirred layer immediately in contact with the sap and the external solution respectively;

$$y_o = S_o a \qquad \text{and} \qquad y_i = S_i(1 - \alpha)x$$

where S is the partition coefficient of the molecule between water and the non-aqueous protoplasm. Whence

$$\frac{dx}{dt} = k_4[S_o a - S_i(1 - \alpha)x]$$

When $1 - \alpha$ is constant the expression can be integrated with a constant to give

$$\int dt = k_4 \int \frac{dx}{S_o a - S_i(1 - \alpha)x}$$

or

$$t = \frac{1}{S_i(1 - \alpha)k_4} - \log\,[S_o a - S_i(1 - \alpha)x] + I$$

---

[19] The derivative $\left(\frac{\partial x}{\partial a}\right)_t$ could, of course, be obtained and the values of B and C substituted. This would serve no useful purpose, however, and will be omitted.

[20] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529.

[21] Longsworth, L. G., *J. Gen. Physiol.*, 1933–34, **17**, 211.

and evaluating I and putting $\frac{1}{S_i(1-\alpha)k_4} = k_5$

$$t = \frac{1}{k_5}\log\frac{S_o a}{S_o a - S_i(1-\alpha)x}$$

or

$$x = \frac{S_o a}{S_i(1-\alpha)}(1 - e^{-k_5 t})$$

And if $S_o = S_i$,[22] as may well be the case, and $1 - \alpha = 1$, as seems to be the case with H_2S, the integral becomes

$$tk_6 = \log\frac{a}{a-x}$$

as $x = a(1 - e^{-k_6 t})$, where $k_6 = \frac{S_o}{k_5}$.

In both cases

$$\left(\frac{\partial x}{\partial a}\right)_t = \text{constant}$$

Thus this mode of entrance leads to the relationship actually found, that $x \sim a$.

This shows that when H_2S penetrates the protoplasmic surface in molecular form we shall get such linear curves as are found in Figs. 3 and 4.

The question may therefore be raised whether the entrance of H_2S follows the exponential law. To decide this question the constant k has been calculated for Series I and II, according to the relationship

$$k = 2.3\frac{1}{t}\log\frac{a}{a-x}$$

and the results have been plotted in Fig. 5*a*. Examination of the plot shows that k is not constant but that it falls off from a mean value of about 0.150 at the start to about 0.90 at the end of 10 minutes. It appears therefore that the entrance of H_2S does not follow the simple "monomolecular" course. However, if we plot the average "constants" up to 5 minutes against the equilibrium concentration

[22] Since H_2S is a very weak electrolyte in aqueous solution it may be inferred that it will be still weaker in the non-aqueous layer of the protoplasm, and that there will be a constant ratio of molecular H_2S in the aqueous phase to molecular H_2S in the non-aqueous phase. This is called S.

we obtain Fig. 5 *b*. This shows that the average "constant" is reasonably independent of the equilibrium concentration of H_2S in the sea water (which corresponds to a in the above equation). This, of course, is a characteristic of the monomolecular rate. It appears therefore that we may be dealing with such a process, but with interfering

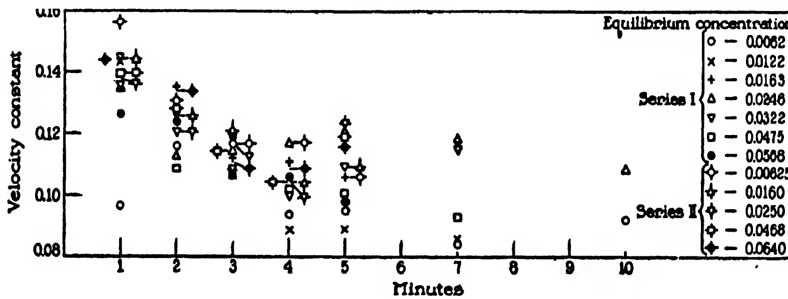

FIG. 5 *a*. Change in the monomolecular velocity constant of H_2S entrance with time. Constant calculated from the formula $k = 2.3 \frac{1}{t} \log \frac{a}{a - x}$, where a is the external concentration of molecular H_2S and x is the total sulfide ($H_2S + S^=$) in the sap.

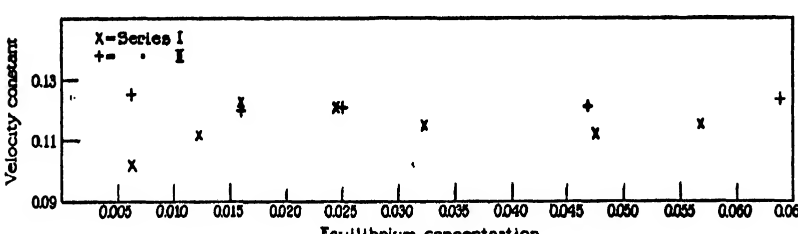

FIG. 5 *b*. Plot of the "average" velocity constant from 1 to 5 minutes for each "equilibrium" concentration of H_2S, the latter being regarded as equivalent to the concentration of molecular H_2S in the external solution.

factors which tend to make the calculation of the constant too high in the early part of the runs.

Referring back to p. 291, we find that the full expression for the relationship between x and a, taking both degree of dissociation and partition coefficients into account, is

$$x = \frac{S_o a}{S_i(1-\alpha)}(1 - e^{-k_0 t})$$

or

$$k_0 t = \log \frac{S_o a}{S_o a - S_i(1-\alpha)x}$$

It will be clear that if $1 - \alpha$ is not unity, x is not a true measure of the internal concentration of molecular H_2S. The pH, however, is decreasing with time and hence $1 - \alpha$ if it changes at all is increasing with time, *i.e.* the term $\frac{a}{a - x}$ calculated on the basis of $1 - \alpha = 1$ is too high at the start, but with the discrepancy decreasing until $(1 - \alpha)$ does actually become unity. Such an adjustment of the value of this term would tend to decrease the early values of k but not the latter ones. However, it is inconceivable that $(1 - \alpha)$ will be constant for a constant value of t, no matter what the value of a.[23] But if it alters with a the relationship $\left(\frac{\partial x}{\partial a}\right)_t$ = constant, cannot be true.

If, however, $S_o \gtrless S_i$ so that the simplified form

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

does not apply, the value of the term $\frac{a}{a - x}$ will be changed.

To illustrate how this might affect the value of k let us consider a case where the external concentration of molecular H_2S is 0.010 normal and the ratio of $S_i \div S_o$ is $1.0 \div 0.75$. For this case arbitrary values have been given to x from 0.001 to 0.005, and the corresponding values of t have been calculated from the formula

$$k = 2.3 \frac{1}{t} \log \frac{S_o a}{S_o a - S_i x}$$

For the first value of x, t is taken as 1 minute which fixes the value of k. Thus the calculations for the case have been idealized to make k constant.

This is compared with the value of k obtained for the case where S_o is taken as equal to S_i, and k has been calculated from the formula

$$k = 2.3 \frac{1}{t} \log \frac{a}{a - x}$$

the value of t being derived from the ideal case. The results have been collected in Table IV.

[23] Unless indeed it is unity for all values of a and t, which seems to be approximately the case of H_2S in the concentration range studied by us.

This shows that failure to take into account the difference between S_o and S_i (the external partition coefficient S_o and the internal S_i) can yield a constant which is decreasing with time in a process which is nevertheless essentially monomolecular. And since S_o and S_i are not changing with time

$$\left(\frac{\partial x}{\partial a}\right)_t = \text{constant}$$

Moreover it will be clear that k is independent of a as is required by the monomolecular relationship.

However, in these experiments it may be argued that the difference of the "partition coefficients" cannot be the cause of the fall of the

TABLE IV

Velocity Constants of H_2S Penetration for Ideal and Non-Ideal Cases

$a = 0.010$

x	Ideal case					Non-ideal case			
	$S_o a$	$S_o a - S_i x$	$\log \frac{S_o a}{S_o a - S_i x}$	t	k	a	$a - x$	$\log \frac{x}{a-x}$	k
0.001	0.0075	0.0065	0.06215	1.000	0.143	0.010	0.009	0.04576	0.105
0.002	0.0075	0.0055	0.13470	2.167	0.143	0.010	0.008	0.09691	0.103
0.003	0.0075	0.0045	0.22185	3.569	0.143	0.010	0.007	0.15490	0.0998
0.004	0.0075	0.0035	0.33099	5.326	0.143	0.010	0.006	0.22185	0.0959
0.005	0.0075	0.0025	0.47712	7.677	0.143	0.010	0.005	0.30103	0.0902

constants because a was actually measured as x at equilibrium when according to the above formulation

$$S_i x = S_o a$$

and since in the adjusted constant of Table IV S_i was taken as unity the value actually measured was $S_o a$. Hence in the calculations from the actual experiments, Fig. 5 *a*, k should have been constant.

It may be supposed also that $S_i < S_o$ so that if S_o is taken as unity $S_i x$ would be less than x. Following the same procedure in using this adjustment we find that its omission would cause the constant to increase with time, the opposite of the observed effect.

There may be other factors than the partition coefficients which should be taken into account.

Thus the constant k_4 in the simplest case, where $S_o = S_i$, is a combination of several factors, including the diffusion constants in the unstirred layers, the thickness of the layers, and the areas of the interfaces.[24] It is scarcely likely that the slight difference in area between the internal and external layers in the same cell can have any effect, but it is entirely possible that the diffusion constants or the thicknesses or possibly both may be different. Just how these will affect the rate is not known. However, for the case of dried collodion, Northrop[25] has shown that

$$\frac{dx}{dt} = \frac{DA}{h}(S_o a - S_i x)$$

where D is the diffusion constant in the collodion, A is the area of the interface, and h is the thickness. Applying this equation to each of the unstirred layers we get for the entrance of H_2S

$$\frac{dx}{dt} = \frac{D_o S_o A_o a}{h_o} - \frac{D_i S_i A_i (1 - \alpha) x}{h_i}$$

This can, of course, be integrated as before if we assume that all the terms are constant but t and x, to give the usual monomolecular formula. And although we do not know the values of any of the constants, as was shown in Table IV, an overall value can be given which will correct the drift in the value of k.

There remains, however, the possibility that the drift in k is due to differences in the permeability of the protoplasm and of the cellulose wall. This possibility is strengthened by the fact that in some experiments of the penetration of CO_2 into dead cells of approximately the same size and shape,[26] where the method of entrance is almost certainly by diffusion, the value of k also fell off with t.

[24] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529, footnote 31. Corresponding to the stirred non-aqueous liquid between the unstirred layers we have in the case of *Valonia* a region between the sap and the external solution interfaces where there is probably some stirring due to protoplasmic movements. The rate of this stirring will, as in the case of the model, affect the rate of entrance, but we may assume that it will be about the same for all cells under the same conditions.

[25] Northrop, J. H., *J. Gen. Physiol.*, 1928–29, **12**, 435. The terms used by Northrop have been translated by us to the terms used in this paper.

[26] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 695.

SUMMARY

The rate of entrance of H_2S into cells of *Valonia macrophysa* has been studied and it has been shown that at any given time up to 5 minutes the rate of entrance of total sulfide ($H_2S + S^=$) into the sap *is proportional to the concentration of molecular H_2S in the external* solution.

This is in marked contrast with the entrance of ammonia, where *Osterhout has shown that the rate of entrance of total ammonia* ($NH_3 + NH_4^+$) does not increase in a linear way *with the increase in the external concentration of NH_3,* but falls off. The strong base *guanidine also acts thus.*

It has been shown that the rate of entrance of H_2S is best explained by assuming that it enters by diffusion of molecular H_2S through the non-aqueous protoplasmic surface.

It has been pointed out that the simple diffusion requires that the rate of entrance might be expected to be monomolecular. Possible causes of the failure of H_2S to follow this relationship have been discussed.

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COMBINATION OF THIOL ACIDS WITH METHYLGLYOXAL

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Lohmann (1) found that glutathione can act as coenzyme for the glyoxalase of tissue extracts and, furthermore, that neither cysteine nor thioglycolic acid can replace glutathione in this process. Jowett and Quastel (2) have evidence that glutathione combines with methylglyoxal and suggest that the resulting compound, under the influence of glyoxalase, breaks to give lactic acid.

The purpose of the present work is to begin a study of the products formed by the interaction of methylglyoxal with thiol compounds. Since phenylglyoxal seems to be completely equivalent to methylglyoxal as regards the action of glyoxalase and since the desired products of phenylglyoxal crystallize more readily than those of methylglyoxal, much of the present work is devoted to the study of the combination of thiol acids with phenylglyoxal. All the products described are easily formed in aqueous or alcoholic solution at room temperature and most of them after separation can be recrystallized easily.

Two types of compound have been isolated by the interaction of methyl- or phenylglyoxal with thiol compounds. The first is formed by simple addition of 1 mole of the glyoxal and 1 of the thiol compound. The second is formed from the two components in the same proportions with elimination of a mole of water. In Table I are listed the thiol compounds whose reaction products with methyl- or phenylglyoxal have been isolated. The type of reaction product, whether simple addition or condensation, is also indicated.

Thiourea, written in its pseudo form, is $HS \cdot C(NH_2):NH$. Thus the only two thiol compounds which give condensation products,

cysteine and thiourea, possess free amino groups in close proximity to the thiol group. Thiosinamine, if written in the pseudo form $HS \cdot C(NHC_3H_5):NH$, has no free amino group, and, in fact, it behaves as the other thiols giving a simple addition compound. A considerable amount of work has been done on the structure of these compounds, but, as the data are not yet conclusive, it is advisable to wait until this is finished before presenting it.

Compounds formed by the direct addition of mercaptans and aldehydes or ketones have been described before. For example, Baumann (3) has made addition compounds of phenyl mercaptan with chloral, pyruvic acid, benzoyl formic acid, and isatin. Fromm and Erfurt (4) described a compound of benzaldehyde and benzyl mercaptan which is so unstable that it cannot be recrystallized

TABLE I

Reaction Products of Methyl- and Phenylglyoxal

	Addition	Condensation
CH_3COCHO	Thioglycolic acid	Cysteine
	" " anilide	Thiourea
	Glutathione	
C_6H_5COCHO	Thioglycolic acid	Cysteine
	" " anilide	Thiourea
	Cysteine betaine	
	Glutathione	
	Thiosalicylic acid	
	Thiosinamine	

from hot benzene. Schönberg and Schütz (5) found that phenanthraquinone, chrysoquinone, isatin, and N-acetylisatin combine at room temperature with various aliphatic and aromatic mercaptans to give addition compounds described as semimercaptols, which dissociate readily on warming.

The compounds described in the present paper are all stable at room temperature. The glutathione and cysteine betaine compounds of phenylglyoxal and the glutathione, cysteine, and thioglycolic acid compounds of methylglyoxal are extremely easily soluble in water, and, when obtained by precipitation with organic solvents, are not definitely crystalline. This group is also quite hygroscopic. The other eight compounds are all quite insoluble in water and can be repeatedly recrystallized from hot organic

solvents such as alcohol or chloroform from which they separate as large well formed crystals with sharp melting points. Of these water-insoluble compounds, those that are derived from thiol acids all dissolve readily in sodium bicarbonate from which they can be precipitated again by acidification. Some of these compounds such as the cysteine phenylglyoxal compound give no nitroprusside test when dissolved in bicarbonate.

In the experimental section there is also described the preparation of cysteine betaine. Both this compound and cystine betaine give beautifully crystalline flavianates which are rather insoluble in water.

EXPERIMENTAL

Methylglyoxal was prepared by the oxidation of acetone with SeO_2 (6, 7) and was kept in 5 M stock solution in both water and absolute alcohol. Phenylglyoxal was prepared from acetophenone by a method described by Kröhnke (8). Since this work was done, a simpler method has appeared (9). Phenylglyoxal was crystallized as the hydrate and kept as such.

If 20 cc. of 0.5 M aqueous methylglyoxal and 0.7 cc. of thioglycolic acid be mixed and allowed to stand several hours, the odor of thioglycolic acid disappears. From this mixture the product could not be isolated in crystalline form and it may be that the addition compound is really a liquid at room temperature. A crystalline mercury salt is, however, easily obtained. To the above mixture after standing 6 or 8 hours, 20 cc. of water are added and about 30 cc. of saturated $HgCl_2$. A white crystalline precipitate forms which would dissolve on further addition of $HgCl_2$. After a day it is heated to dissolve the precipitate and the solution is filtered and cooled. The crystalline product appears as flat hexagons. It is filtered off, washed with much water, and dried *in vacuo* over H_2SO_4. It decomposes at about 220°.

$(C_5H_7O_4S)_2Hg$. Calculated, S 12.16; found, S 12.05

To a suspension of 4 gm. of thioglycolic acid anilide in 20 cc. of water are added 5 cc. of 5 M aqueous methylglyoxal. The anilide dissolves slowly and a new crystalline precipitate appears immediately. After standing 3 hours with occasional shaking, the product is filtered off. It may be recrystallized by dissolving in

15 cc. of hot alcohol, cooling, and slowly adding about 10 cc. of water. A liquid precipitate first appears which soon crystallizes in long needles. After crystallizing again from 30 cc. of cold alcohol by addition of 100 cc. of water and drying, the product shows a melting point of 70°.

$C_{11}H_{13}O_3NS$. Calculated, S 13.38, N 5.86; found, S 13 49, N 6.09

To 2 gm. of glutathione in 10 cc. of water there are added either 2.5 cc. of 5 M aqueous methylglyoxal or 1.2 gm. of phenylglyoxal. The phenylglyoxal slowly dissolves. If necessary the solution is filtered and after standing a few hours the solution volume is reduced to about 8 cc. by evaporation in a vacuum desiccator. It is then poured into about 300 cc. of absolute alcohol. In each case a white precipitate appears which rapidly becomes sandy on standing. This is filtered off and washed with absolute alcohol. It may be dissolved in a small volume of water and reprecipitated in the same way. It is quickly set *in vacuo* over H_2SO_4 and after being dried thoroughly it can be exposed to the air without becoming gummy.

$C_{13}H_{21}O_8N_3S$. Calculated, S 8.44, N 11.08; found, S 8.51, N 11.03
$C_{18}H_{23}O_8N_3S$. " " 7.26, " 9.52; " " 7.08, " 9.27

If cysteine hydrochloride is added to an aqueous or alcoholic solution of methylglyoxal, the solution gradually turns brown, becoming very dark in about 6 hours. Addition of potassium acetate hastens this process, while addition of acid retards it. That oxygen from the air is not involved in this change is clear from the fact that the same mixture made up under nitrogen becomes brown at the same rate. A mixture of 2.4 gm. of cysteine hydrochloride, 10 cc. of absolute alcohol, and 3 cc. of 5 M methylglyoxal in absolute alcohol is allowed to stand overnight. The brown solution is filtered from any amorphous material and excess cysteine, and to the filtrate several volumes of acetone are added. An amorphous precipitate forms. No way has been found of crystallizing this brownish product. It is filtered off and dried. On exposure to air it takes up moisture and becomes gummy.

$C_6H_9O_3NS$. Calculated, S 18.28, N 8.00; found, S 17.34, N 7.77

An attempt to get a purer product by allowing an aqueous solution of methylglyoxal and cysteine hydrochloride to stand under

hydrogen overnight, then precipitating an amorphous mercury salt from a large volume of the aqueous solution, filtering this off, and removing the mercury by H_2S, evaporating the resulting solution to dryness, extracting the residue with a small volume of acetone, precipitating with ethyl acetate, dissolving the precipitate in absolute alcohol, and finally precipitating with acetone still gave an amorphous, brownish, hygroscopic product. Its analysis was, however, better. Found, S 18.29, N 8.06.

A solution of 2.5 gm. of thiourea in 5 cc. of water and 5 cc. of 5 M aqueous methylglyoxal is set on ice. After a day a slight amount of a reddish precipitate has formed. This is filtered off and the solution again set on ice for 2 days. Occasional scratching hastens the crystallization. The product may be recrystallized from a small volume of water. M.p. 159°.

$C_4H_6ON_2S$. Calculated, S 24.60, N 21.52; found, S 24.59, N 20.91

To a suspension of 15 gm. of phenylglyoxal hydrate in 100 cc. of water are added 10 cc. of thioglycolic acid, and the mixture is stirred. The phenylglyoxal dissolves in about 15 minutes and a new crystalline deposit slowly forms. It is set on ice for 2 days; then the crystalline deposit is filtered off. This may be dried and recrystallized from hot $CHCl_3$. After standing several days, the crystals are filtered off and dried *in vacuo*. M. p. 118°.

$C_{10}H_{10}O_4S$. Calculated, S 14.16; found, S 14.20

The thiosinamine compound is prepared similarly from 2 gm. of thiosinamine and 2 gm. of phenylglyoxal hydrate in 25 cc. of water. The product may be dissolved in 30 cc. of alcohol and precipitated with water or it may be dissolved in 500 cc. of 25 per cent hot alcohol from which it crystallizes on cooling. After drying it shows a melting point of 108°.

$C_{12}H_{14}O_2N_2S$. Calculated, S 12.80, N 11.20; found, S 13.23, N 10.94

In 60 cc. of alcohol are mixed 3.4 gm. of thioglycolic acid anilide and 2.6 gm. of phenylglyoxal hydrate. The anilide dissolves slowly and a new crop of crystals appears. Set on ice for 2 hours. The product is recrystallized twice from 150 cc. of hot alcohol. M. p. 149°.

$C_{16}H_{15}O_3NS$. Calculated, S 10.63, N 4.65; found, S 10.35, N 4.79

The thiosalicylic acid compound is made as is the preceding from an equimolecular mixture of the components. It is recrystallized from alcohol. M. p. 142°.

$C_{15}H_{12}O_4S$. Calculated, S 11.11; found, S 10.81

A solution of 10 gm. of cysteine hydrochloride in 100 cc. of water is stirred with 7.5 gm. of phenylglyoxal hydrate until the latter dissolves. After being set on ice for 2 days, the crystalline precipitate is filtered off, washed with water, sucked dry, and recrystallized from 100 cc. of hot alcohol. M. p. 141°.

$C_{11}H_{11}O_3NS$. Calculated, S 13.49, N 5.91; found, S 13.55, N 5.93

The thiourea compound is made exactly as the cysteine compound just described, 7.5 gm. of thiourea being used instead of the cysteine hydrochloride. The product melts at 222°.

$C_9H_8ON_2S$. Calculated, S 16.66, N 14.58; found, S 16.26, N 14.44

A solution of 2.7 gm. of phenylglyoxal hydrate in 20 cc. of absolute alcohol is stirred up with 3 gm. of cysteine betaine. The betaine dissolves in about an hour and the mixture is allowed to stand overnight. It is then a perfectly clear solution and is poured into 2.5 liters of ethyl acetate. A white precipitate forms which under the microscope looks pebbly but not crystalline. The product after standing a day, during which it becomes more granular, is filtered off, washed with ethyl acetate, and quickly set *in vacuo* over H_2SO_4 as it tends to become gummy in moist air.

$C_{14}H_{19}O_4NS$. Calculated, S 10.77, N 4.71; found, S 9.52, N 4.32

The preparation of cystine betaine offers no difficulties, provided a pure cystine be used and the temperature be kept low during methylation. A flask containing 26 gm. of cystine in 200 cc. of water is immersed in ice water and a mechanical stirrer is inserted. Two dropping funnels are set up, one with 110 cc. of 6.5 M KOH solution and the other with 67 cc. of freshly distilled dimethyl sulfate. Enough KOH solution is run in to dissolve the cystine; then the dimethyl sulfate and the KOH solution are dropped in simultaneously with the stirrer operating. The addition should last 20 to 30 minutes. After standing another 20 minutes at

room temperature 15 cc. of glacial acetic acid are added and the clear solution is evaporated *in vacuo* to small volume. A heavy crystalline precipitate separates. Add 500 cc. of alcohol and filter from the precipitate of potassium methyl sulfate. To the filtrate add 2.5 liters of acetone and let stand a day. The crystalline product is filtered off and dissolved in about 100 cc. of water and reprecipitated with 500 cc. of acetone. After drying *in vacuo* over H_2SO_4 about 15 gm. are obtained.

$C_{12}H_{24}O_4N_2S_2 \cdot H_2O$.	Calculated.	S 18.71, N 8.19, C 42.10, H 7.66
	Found.	" 18.80, " 8.22, " 41.02, " 7.58

Cystine betaine can be reduced by the usual method for the reduction of cystine, in 4 M HCl with tin. After removing the tin with H_2S, the solution is evaporated *in vacuo* to small volume and the product precipitated with acetone as with cystine betaine.

$C_6H_{13}O_2NS \cdot 2H_2O$. Calculated, S 16.08, N 7.04; found, S 16.38, N 6.98

Both cystine betaine and cysteine betaine are easily soluble in water and both give crystalline, insoluble Ag salts. The latter gives intensely colored brown complexes with cobalt salts in alkaline solutions, just as cysteine does. Both give quite insoluble salts with flavianic acid. 1 gm. of either betaine in 25 cc. of water is mixed with a solution of 2 gm. of flavianic acid in 50 cc. of water and the mixture is set on ice overnight. The yellow crystalline precipitate is filtered off and recrystallized from hot alcohol, about 200 cc. being used for the cystine compound, while only 50 cc. are needed for the more soluble cysteine compound. The cystine betaine flavianate melts at 230° and the cysteine compound melts at 210°.

$(C_{12}H_{24}O_4N_2S_2) \cdot (C_{10}H_6O_8N_2S)_2$.	Calculated.	S 13.44, N 8.82
	Found.	" 13.17, " 8.38

For the corresponding cysteine compound, $(C_6H_{13}O_2NS) \cdot (C_{10}H_6O_8N_2S)$

Calculated, S 13.41, N 8.80; found, S 13.30, N 8.69

SUMMARY

It has been shown by the direct method of isolation that both methyl- and phenylglyoxal combine with thiol acids. The prod-

ucts consist of 1 mole of the glyoxal and 1 of the thiol acid. Two types of product are described, the first formed by simple addition, the second by simple addition with elimination of a mole of water. The second type of compound seems to be formed by those thiols which possess a nearby amino group such as cysteine.

The preparation of cystine betaine and cysteine betaine is described.

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DERIVATIVES OF KERATIN

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In an earlier paper the authors (1) expressed the view that keratins are fibrous proteins whose characteristic properties are essentially determined by the S—S groups of cystine which act as very firmly established cross links uniting the elementary fibers of polypeptide chains. This view was based on the action of certain alkaline reductants which could be shown to reduce disulfides and convert keratin into an amorphous protein soluble in weak alkalies, digestible by true proteases, and in which the sulfur is in the sulfhydryl state. The important rôle of the disulfide bond had already been emphasized by Speakman and Huist (2) and Astbury (3) on the basis of their chemical and physical studies, including x-ray diffraction patterns of keratins.

Since keratins have a very high percentage of disulfide sulfur (10 to 15 per cent cystine) and may readily be reduced to sulfhydryl proteins by alkaline thioglycolate, we realized that this reduced protein might be a useful material for study as an example of a sulfhydryl protein. We were particularly interested in studying the properties of derived proteins formed by substitution in the sulfhydryl group by reaction with organic halogen compounds according to the following scheme.

$$A—SH + R—X \rightarrow A—S—R + HX$$

This reaction occurs with great ease at neutral to mildly alkaline reaction, and has already been applied to proteins for different reasons by Mirsky and Anson (4) and Goddard and Schubert (5). Although organic halogen compounds can react with amino groups according to Michaelis and Schubert (6), we will show that it has been possible under certain conditions to substitute completely

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the sulfhydryl groups of the protein of reduced wool keratin, without substituting amino groups.

The proteins studied are the sulfhydryl protein obtained by the alkaline thioglycolate reduction of wool, this protein after it has been reoxidized to the disulfide form, and the substituted proteins formed by reaction of the sulfhydryl protein with iodoacetic acid, iodoacetamide, iodoethyl alcohol, and α-bromopropionic acid, at mildly alkaline reaction. The disulfide protein may be formed by reoxidation of the sulfhydryl protein at neutral reaction by the air or ferricyanide. As to the nomenclature of these proteins, we may designate the reduced keratin as kerateine, and the reoxidized, amorphous kerateine as metakeratin. By reaction of kerateine with iodoacetate we obtained a carboxymethylkerateine; with α-bromopropionic acid, α-carboxyethylkerateine; with iodoethyl alcohol, hydroxyethylkerateine; with iodoacetamide, carbamylmethylkerateine.

None of the derived keratins has been obtained in a strictly pure state, nor may they be considered as chemical entities. It is not even likely that the keratin of a single hair is a homogeneous chemical substance. Furthermore, it should be borne in mind that the reduction of the disulfide group by thioglycolate is a reversible reaction, and its completeness depends on the excess of the reductant applied, and that the reaction with the halogen compounds can proceed only to that extent to which the S—S group has been previously reduced. In the preparation of a substituted protein the non-reduced, non-substituted residue can be determined as cystine by applying the Folin-Marenzi method to the hydrolysate. The substituted sulfur does not react in this method.

The analytical data for these proteins and wool are presented in Table I. It will be seen that the S and N are similar to the values of wool, and secondly, that nearly all the cystine has been reduced and substituted.

Proceeding to the isoelectric points and solubilities of these derived proteins, it should be interesting to compare native wool with the derived proteins in this respect. There is no agreement as to the isoelectric point of native wool. Elöd and Silva (7), from swelling experiments and changes in the pH of solutions in which wool was allowed to come to equilibrium, determined the isoelectric point to be 4.9. This is in agreement with the recent result

TABLE I

Analysis of Keratin Derivatives (Per Cent, Except Column of Isoelectric Points)

Protein	Total S	Total N	Cystine	Cystine S	Amino N	Isoelectric point	Solubility
Native wool	3.35 3.33	16.29	11.6	3.12		4.9(?)	Insoluble in M NaOH or HCl
Kerateine	3.34	16.50	12.2*	3.24		4.6–4.9	Soluble in 0.1 M Na_2CO_3 and NH_4OH; insoluble in N/30 HCl
Metakeratin	3.37	16.50 16.54	12.2, 12.1	3.25	0 81, 0.79	4.5–4.7	Soluble in 0.1 M Na_2CO_3 and NH_4OH; insoluble in N/30 HCl
Carboxymethylkerateine, unfractionated†	3.31		1.42	0.38		3.8–4.3	Soluble in 0.1 M sodium acetate or N/30 HCl
Carboxymethylkerateine, Fraction A	3.33 3.29	16.32	0.80	0.21	0.81, 0.78	3 7–4.3	Soluble in 0.1 M sodium acetate and M/30 HCl
Carboxymethylkerateine, Fraction B	4.50 4.55	14.95	0.97	0.26	0.50	3.0–3.3	Soluble in 0.1 M sodium lactate and lactic acid
Carboxymethylkerateine‡	2.00 2.00	15.90	1.73	0 46	0.80, 0.76	3.3–3.6	Soluble in 0.1 M sodium acetate and M/30 HCl
Carbamylmethylkerateine	2.78 2.84	17.35§	2.74 2.40	0.75		5.0–5.3	Insoluble in sodium acetate; soluble in M/30 HCl
α-Carboxyethylkerateine	2.12 2.15 2.17	15.84	1.35	0.36	0.76	3.1–3.7	Soluble in 0.1 M sodium acetate and M/30 HCl
Hydroxyethylkerateine	2.73 2.76 2.77	15.50 15.30	2.66	0.71		4.5–4.9	Soluble in 0.1 M Na_2CO_3 or NH_4OH, insoluble in M/30 HCl or 0.1 M sodium acetate

* Total cystine = 12.2; of this 88 per cent was in the form of cysteine.

† This was not the preparation from which Fractions A and B were prepared.

‡ This preparation was allowed to react with iodoacetate for 40 hours at pH 9.0 to 9.5. Notice the conspicuous loss of sulfur.

§ The increased N content is due to the amide N of iodoacetamide.

of Dumanski and Dumanski (8) of 4.9 obtained by deflections of wool fibers in an electrical field. Speakman (2) denies that wool has any distinct isoelectric point and acknowledges only a wide isoelectric zone of 5.0 to 7.0. Harris (9) has determined the isoelectric point of wool as 3.4 by cataphoresis of wool particles. This disagreement makes the comparison with the derived proteins difficult. In these derived proteins there is no difficulty in determining the isoelectric point with the flocculation method (10). Kerateine and metakeratin prepared from wool have isoelectric points in the region 4.6 to 4.7. Carboxymethylkerateine and α-carboxyethylkerateine have much lower isoelectric points, as would be expected from introduction of the carboxyl groups. Carbamylmethylkerateine has a slightly higher isoelectric point, while hydroxyethylkerateine has the same isoelectric point as kerateine. The change in isoelectric points and solubilities of these derived proteins are what would be expected from the chemical nature of the substituted groups.

A comparison of the solubilities of these compounds shows the following features. Wool is insoluble in all acids and bases except in so far as it is hydrolyzed. Wool dissolves readily in alkaline solutions of NaCN, Na_2S, and thioglycolate (1), but in all these cases the fibrous pattern is irreversibly destroyed. Metakeratin and kerateine of wool are dissolved readily in weak (0.1 M) NaOH, Na_2CO_3, and NH_4OH. They are insoluble in sodium acetate, water, and neutral salts, and slightly soluble in dilute HCl. Stable neutral solutions containing several gm. of metakeratin in 100 cc. may be obtained by dialyzing the alkaline solution. These solutions are precipitated by traces of acetic or other acids, and once precipitated are as difficulty soluble as the undialyzed metakeratin. Carboxymethylkerateine and carboxyethylkerateine are very readily soluble not only in solutions of $NaHCO_3$ and dilute HCl, but even in a solution of sodium acetate. Carbamylmethylkerateine has the same solubility in alkalies as kerateine, but is much more soluble in dilute HCl than is kerateine. The solubility of hydroxyethylkerateine is similar to kerateine.

Determinations of free amino N of some of our preparations were performed by the method of Van Slyke (11) in the constant volume apparatus. The main purpose of this analysis was to show whether under the conditions of our work a substitution of the H

atoms of the amino group occurred by reaction of kerateine with iodoacetate, which could be imagined according to the results of Michaelis and Schubert with simple amino acids (6). The figures show definitely that no detectable substitution of amino groups occurred, even in a period of 40 hours at a pH about 9.0 to 9.5. This is of interest to the general problem as to the point of attack of iodoacetate on the enzymes which it poisons.

As regards the percentage of amino nitrogen, Van Slyke and Birchard (12) found that in several proteins the free amino N was equal to half the lysine nitrogen, with the exception of gliadin in which it is much higher. The free amino N, as determined by the Van Slyke method on metakeratin and several derived kerateines, is somewhat higher than would be expected from the published values of the lysine content of wool of 2.2 (13), 2.3 (14), and 2.8 (15) per cent. If we take the nitrogen content of wool as 16.5, then the percentage of free amino nitrogen referred to total nitrogen should be 1.8 to 2.3 per cent, depending upon the value assumed to be the correct lysine value. We find that 4.7 to 4.9 per cent of the total N is free amino N in our preparations.

It is not likely that the whole substance of a hair, even disregarding any pigment, represents a single homogeneous protein. The usual method of separating a natural protein mixture into its component parts is the fractional precipitation with salts. Such a method obviously cannot be used for an insoluble protein such as keratin. Even for kerateine and metakeratin the solubility is too low for an attempt of fractionation. But the much wider range of solubility of some of the derived proteins makes possible fractional precipitation. Carboxymethylkerateine was fractionated with ammonium sulfate from solution of the protein in 0.1 M sodium acetate. The fractionation led to two fractions; one which contained the major bulk of the original protein and had a similar content of S, N, and amino N as the original protein; and a second small fraction, Fraction B, which showed higher S and lower N and amino N content. Fraction A is completely precipitated from solution by 35 per cent saturation with $(NH_4)_2SO_4$ and gives a compact, cheese-like precipitate. This fraction is completely insoluble at its isoelectric point, it does not dissolve appreciably in dilute lactic acid or sodium lactate, but does dissolve readily in dilute sodium acetate or dilute HCl. The other

fraction, Fraction B, is soluble in 35 per cent saturated $(NH_4)_2SO_4$, but is completely precipitated as a very sticky, non-flocculent precipitate at 60 per cent saturation. It is not completely insoluble even at its isoelectric point and readily dissolves in an excess of dilute lactic acid or even in sodium lactate. The high sulfur content of Fraction B indicates that the native keratin probably consists of two fractions, and not that the substitution leads to two different products.

One of the characteristic properties of keratin is its complete resistance to digestion by trypsin and pepsin. But all of the proteins prepared from wool, either as sulfhydryl, disulfide, or substituted proteins, are as readily digested by trypsin and pepsin as are typical proteins. The resistance of keratin to digestion is not dependent upon the disulfide state as such, since metakeratin is readily digested, but upon the fibrous property of the keratin, as brought about by the spacial arrangement of the S—S bonds. Once this bond has been broken, the protein becomes digestible by true proteases no matter what other reactions the sulfur may undergo.

As regards the oxidation and reduction of S of these proteins, native keratin may only be reduced at a pH of 10 or higher, but metakeratin is reduced by the same agents at a pH of 7 to 8. Kerateine is readily oxidized to metakeratin, even dialysis of the protein suspended in acetate buffer at pH 4.6 leads to nearly complete oxidation in 3 days. In an alkaline solution oxidation is much more rapid. Within the short time required to dissolve kerateine in ammonia, to filter, and to reprecipitate (20 to 30 minutes), complete oxidation takes place. Such a high degree of autoxidizibility is as a rule not encountered in other sulfhydryl proteins according to A. E. Mirsky (personal communication).

EXPERIMENTAL

The "Parent" Protein—For the preparation of the substituted proteins a single batch of wool protein, essentially metakeratin still containing some unoxidized kerateine, was used. This protein is spoken of as the "parent" protein. It was prepared essentially as described by Goddard and Michaelis (1). 100 gm. of defatted but otherwise native, untreated wool are dissolved by mechanical shaking in 2 liters of 0.5 M disodium thioglycolate (if

the pH is maintained between 11 to 12, this takes about 3 hours). The undissolved residue is removed by centrifugation followed by filtration, the filtrate is precipitated with acetic acid, collected on the centrifuge, washed with acetone, and ground in a mortar with acetone. After freeing from acetone by a vacuum, the protein suspension is dialyzed in cellophane tubes until free of salts and thioglycolic acid. The dialyzed protein is washed with acetone and dried in a vacuum desiccator. It is obtained as a fine white powder containing about 4 per cent moisture, nearly insoluble in water, but slightly soluble in acids, and very (but slowly) soluble in alkali. This powder has essentially the same content of nitrogen, sulfur, and cystine as the wool from which it is prepared. It becomes to a great extent reoxidized on preparation. A typical preparation, hydrolyzed and analyzed by the Mirsky and Anson (4) modification of the Folin-Marenzi method, contained 12.3 per cent of total cystine (cystine + cysteine) and 3.17 per cent of cysteine. So, the sulfur, which had been practically completely reduced, has been reoxidized during the preparation to 75 per cent.

Kerateine—10 gm. of parent protein were freshly reduced under oxygen-free nitrogen with 4.65 gm. of thioglycolic acid at a pH just pink to phenolphthalein for 2 hours. This protein was then precipitated with trichloroacetic acid, collected on the centrifuge, washed with acetone, transferred to a mortar, and ground three times with acetone and three times with acid acetone, and dried in a vacuum desiccator.

Metakeratin—The parent protein, as stated above, is a mixture of kerateine and metakeratin. An attempt to convert this completely to metakeratin by oxidation with 3 per cent H_2O_2 at a pH of about 8.5 led to a loss of cystine, and on acidification H_2S could be smelled. The cystine[1] content before oxidation of the protein was 12.27 and after 10.51 per cent.

Metakeratin without loss of sulfur may be prepared from kerateine by oxidation in mildly alkaline solution with potassium ferricyanide. 15 gm. of parent protein were suspended in 300 cc.

[1] The cystine content here includes the cysteine, for the protein hydrolysate is oxidized, *while still acid*, with 3 per cent H_2O_2. Then the solution is reduced with sulfite and analyzed by the Folin-Marenzi procedure.

of H_2O and M NaOH was added with constant stirring until a faint pink color developed with phenolphthalein; after some time the protein was completely dissolved. 0.2 M potassium ferricyanide was added with a burette until the yellow color did not disappear instantly. The protein was dialyzed until acid to litmus; this gave an opalescent solution free of precipitate, but easily coagulated by traces of acetic acid. The protein was precipitated with dilute acetic acid, washed with acetone, and dried in a vacuum desiccator.

Carboxymethylkerateine and Its Fractionation—50 gm. of parent protein were suspended in 1000 cc. of water, 20 cc. of M NaOH added, and nitrogen led through the mixture. 32.5 gm. of thioglycolic acid were neutralized to phenol red and 250 cc. of 3.4 M phosphate buffer, pH about 7.4, added to it; this solution was added to the protein. After 3 hours 75 gm. of recrystallized iodoacetic acid, neutralized to phenol red, were added to 250 cc. of the same buffer, and added to the protein-thioglycolate solution. After 2 hours the protein was precipitated by adding enough solid $(NH_4)_2SO_4$ to give a 50 per cent saturated solution. The protein was filtered off, washed with 50 per cent saturated $(NH_4)_2SO_4$, and redissolved in 1500 cc. of H_2O + 20 cc. of M NaOH. It was again reduced with thioglycolic acid and allowed to react with iodoacetic acid exactly as in the procedure above. The protein was precipitated with $(NH_4)_2SO_4$ at 50 per cent saturation and collected on a centrifuge, and the supernatant liquid was discarded. This protein was then dissolved in 1200 cc. of 0.1 M sodium acetate and fractionated by precipitation at 35 per cent saturation with $(NH_4)_2SO_4$. A second fraction was obtained from the filtrate by 60 per cent saturation with $(NH_4)_2SO_4$. The fractions were purified by repeating this procedure several times. The first fraction, Fraction A, represents the bulk of the material; the final yield was 30 gm. of dry protein. The other fraction, Fraction B, gave a yield of only 2 gm. of dry protein.

Another preparation of carboxymethylkerateine was prepared from freshly reduced parent protein by treatment with iodoacetate at pH 9.0 to 9.5 for 40 hours, the iodoacetate being added in intervals and to a large excess, in an attempt to substitute the amino groups as well as the —SH groups. The analysis shows that no detectable substitution of the free amino groups occurred,

and that the long alkaline treatment caused a loss in sulfur, as is shown in Table I.

α-Carboxyethyl-, Carbamylmethyl-, and Hydroxyethylkerateine—These three preparations were prepared in almost identical manner. 10 gm. of parent protein were suspended in 100 cc. of H_2O, for each preparation, and sufficient M NaOH to give a light pink color with phenolphthalein, without attempting to dissolve the protein completely. 4.65 gm. of thioglycolic acid neutralized to phenolphthalein were added to each, and the flasks stood for 3 hours under a stream of oxygen-free nitrogen. Then either 9.1 gm. of α-bromopropionic acid (neutralized to phenolphthalein) or 11.0 gm. of iodoacetamide, or 9.5 gm. of freshly distilled iodoethyl alcohol were added. From time to time additional NaOH was added to maintain the pH at a light pink to phenolphthalein. After 2 hours the preparations made with iodoacetamide and iodoethyl alcohol were precipitated with acetic acid, washed with acetone, and then the suspensions dialyzed against distilled H_2O for 3 days. The preparation with α-bromopropionic acid stood overnight, was reduced in a similar manner as above, and treated a second time with bromopropionic acid for 4 hours. The protein was then dialyzed for 3 days, precipitated with trichloroacetic acid, washed with acetone, and dried in a vacuum desiccator. The analytical results are listed in Table I.

Isoelectric Points—The isoelectric points were estimated by the method of Michaelis and Rona (10). The proteins with the higher isoelectric points were dissolved in 0.1 M sodium acetate and precipitated with 0.1 M acetic acid (final concentration of acetate ion was 0.01 M), while the proteins of the isoelectric zone not covered by the pH range of acetate buffer were dissolved in 0.1 M sodium lactate and precipitated with lactic acid. pH of the mixtures was determined with the glass electrode.

Digestion—Purified trypsin (16) and Fairchild's pepsin were used. The soluble proteins were digested with great rapidity (no precipitate with trichloroacetic acid or sulfosalicylic acid after 10 to 15 minutes at 37°). Those proteins not entirely soluble at this pH were digested more slowly but at a rate comparable to other coagulated proteins.

Methods of Analysis—All the proteins were analyzed for nitrogen by Teorell's (17) micro-Kjeldahl method, for sulfur by the method

of Elek and Hill (18), and for cystine by the Folin and Marenzi (19) procedure, with a Zeiss-Pulfrich photometer. If the protein contained both cystine and cysteine the acid hydrolysates were oxidized with 3 per cent H_2O_2, and then the excess H_2O_2 was removed by the sulfite used in the cystine reduction. Cysteine was analyzed for by the iodoacetate method of Mirsky and Anson (4). Amino nitrogen was determined by the method of Van Slyke (11) in the constant volume apparatus. The proteins were dissolved by grinding in a mortar with 10 cc. of 0.1 M NaOH and diluted to 50 cc. 5 cc. of solution containing 20 mg. of protein were used for analysis; the nitrite and protein were added prior to the addition of the acid. The time of reaction with nitrous acid was 30 minutes.

SUMMARY

Keratin of wool is reduced by sodium thioglycolate to kerateine. This can be oxidized to metakeratin which differs from native keratin by its amorphous character, solubility in alkali, and by its digestibility with pepsin or trypsin.

The H of the —SH group of kerateine was substituted by treatment with iodoacetate, α-bromopropionate, iodoacetamide, and iodoethyl alcohol. The derived proteins thus obtained differ distinctly in their solubilities and in their isoelectric points. They are all digested by pepsin or trypsin. No detectable substitution of amino hydrogen occurred during the treatment, and under proper treatment no loss of sulfur occurred. The derivative obtained with iodoacetic acid is soluble enough to allow the attempt of a fractionation with ammonium sulfate. This led to two fractions differing widely in solubility and sulfur content.

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AN IMPROVED SELF-RECTIFYING GAS X-RAY TUBE

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To meet many of the present-day problems of crystal analysis it is essential to have more intense x-ray beams than can be obtained from commercially available tubes. In most instances the bodies of home-made tubes built to provide such beams are of metal and they operate with continuous pumping. They either can have hot-wire cathodes or they can be of the original gas type. It has been our experience that gas tubes are more satisfactory for routine crystal experimentation. This is partly because they require a relatively poor vacuum, but it is an additional advantage that with them sputtering and vaporization of the cathode cannot lead to an impure spectral output. Early gas tubes required a rectified high voltage input. Several recent designs are self-rectifying and the following tube is an improvement over one[1] of these.

The energy that could be put through our earlier model was limited by its wax seals; much usable intensity also was lost because of the considerable distance between the focal spot and the accompanying slit system. In the present tube all wax seals have been replaced by lead sheet and fuse wire[2] seals and interchangeable slit systems have been built into the head so that the first slit is virtually in contact with the target. Besides increasing the beam intensity, built-in slits have an added value in limiting to a large extent the amount of stray radiation from a tube. With powerful sources this is an important consideration for the protection of both the photographic films and the health of the experimenter.

If the stated dimensions are adhered to, these tubes are self-rectifying when passing several kilowatts. Their useful power rating

[1] R. W. G. Wyckoff and J. B. Lagsdin, Radiology **15**, 42 (1930).

[2] P. Kirkpatrick and P. A. Ross, R. S. I. **4**, 645 (1933).

depends entirely on what their targets can stand; this in turn is conditioned partly by the thermal conductivity of the target metal, partly by its melting point and partly by the area of the focal spot. With a very finely-focused beam we have drilled a hole through a water-cooled copper target when using about one kilowatt; with a beam too broad to be valuable in diffraction work about five kilowatts have been safely

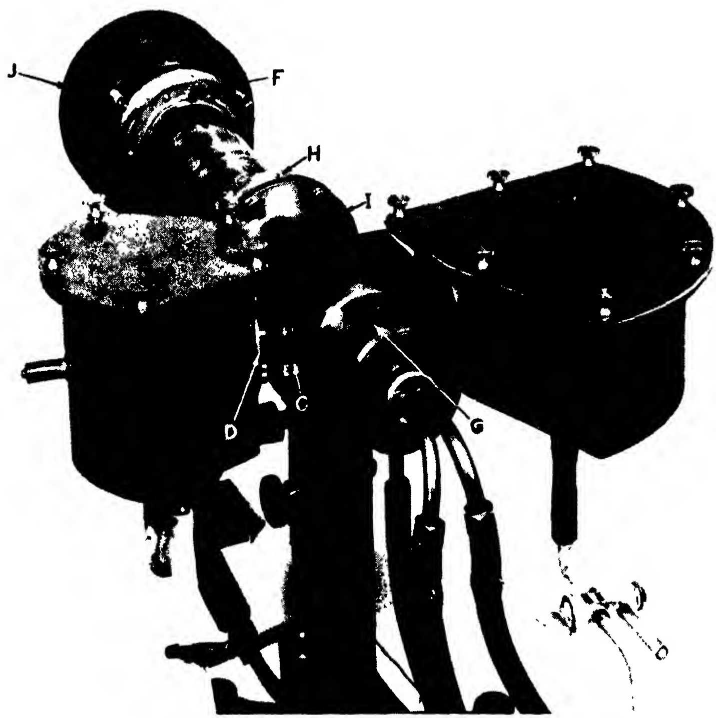

FIG. 1. A photograph of the x-ray tube with two attached diffraction cameras. The labeled parts are described in the text or in the legends to the next figure.

put through these tubes. It would seem that still more intense useful beams can only be obtained by introducing moving targets.

The general appearance of the present tube is shown in Fig. 1; details of the metallic head are indicated in the scale drawing of Fig. 2. For most purposes it is convenient to have the diffraction cameras permanently attached to the slit systems. Two such cameras, of

different radii, are shown at A and B of Fig. 1. They are separated from the interior of the x-ray tube by 0.0005 inch thick aluminum foil (a of Fig. 2b) and can be evacuated or filled with helium or hydrogen to minimize air scattering.

One of the removable slit systems is shown schematically in Fig. 2b. The sizes of the three slits contained in the interchangeable insert b will depend on the type of sample and the part of the diffraction pattern under investigation. It must be remembered that the foil a

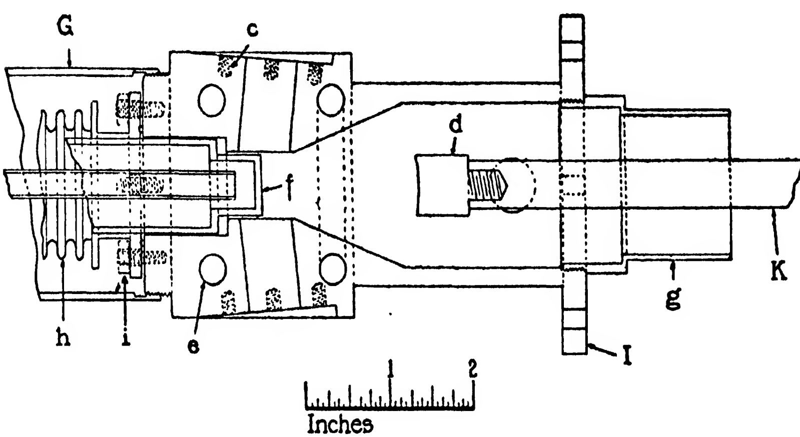

FIG. 2a. A scale drawing of a section through the target end of the tube. The head, milled and turned from a rod, is cooled by water flowing through the channels e; the slit systems are fastened by bolts screwing into c. The bolts (i) which attach the target assembly to the tube body are also shown; the separate water leads for the target can be seen in Fig. 1. As in the earlier tube the guard ring g screws separately into the head. The position of the cathode rod K and its interchangeable aluminum tip d indicates the approximate cathode-target distance for proper focusing. The Pyrex insulator E is bolted to the tube flange I with the clamp H (Fig. 1).

which seals the tube can act as a weak secondary source; this must be taken into account if reflections at very small angles are to be recorded.

Except that a short piece of sylphon tubing is used in the cathode end for fine adjustment, its construction, including that of the cooling fins J, is like that of the early model. A focal spot of the required area is obtained by screwing the cathode rod in or out. In the tube pictured in Fig. 1 the shape of the spot is followed by replacing one of the slit systems by a single pinhole slit to provide a pinhole camera; in

more recent tubes we have attached such a camera permanently to the top of the tube itself. When a focal spot of approximately the desired size has been obtained, it is centered on the target and finely focused by the three screws F. This is conveniently done with a long rubber wrench while the tube is operating. The target itself, f, is moved slightly in and out (by turning G and thereby compressing or expanding the sylphon h) until the beams through the two slit systems

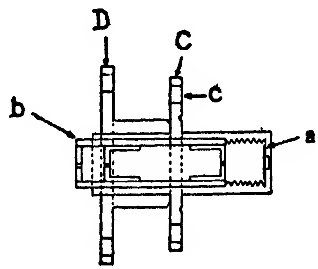

FIG. 2b. A schematic drawing of one of the interchangeable slit systems. It is bolted to the camera through the flange D, to the tube through C.

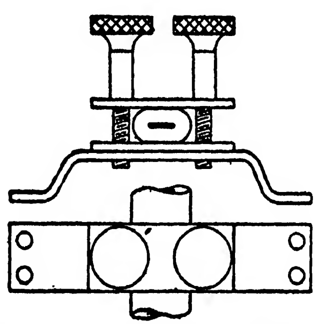

FIG. 3. Two views of the simple clamp used as a variable leak.

are of maximum intensity. Adjustment is then complete. If the targets of different metals are made strictly interchangeable it should not be necessary to alter this adjustment even when irregular operation shows that a new cathode tip (d) must be inserted.

The glass insulator E consists of two conical drain connections of Pyrex fused together. It is very important that these insulators be carefully annealed; otherwise they invariably fracture either on assemblage or soon after the tube is put in use. Usually we have found

it necessary to true up the ends of these glass insulators by grinding; wire washers are placed not only as seals between the insulator and the metal parts of the tube but also as packing between them and the assembly clamps (such as *H*). After two or three days operation of a new tube it is necessary to take up cautiously on all these seals.

One of the greatest advantages gained by eliminating all wax seals is the simplification possible in the pumping system. These tubes will operate very constantly by linking them directly to a good laboratory oil pump, without diffusion pump and without any automatic gas control. A thoroughly satisfactory leak,[3] and one which is in fact better than any of the several others we have tried, can be made by squeezing heavy nitrometer rubber tubing in a broad clamp of the sort shown in Fig. 3. In using such a clamp it is of course necessary to open it every few days to prevent sticking of the inner surfaces of the rubber tubing. A considerable deposit of fine powder accumulates in the interior of tubes passing large currents. This deposit can adsorb large amounts of gas which may subsequently be freed under the influence of a discharge. For this reason it is usually best to disassemble the head and scrape it clean if for any reason it is necessary to readmit air to the system after the tube has been run for some time with a large power input.

[3] We are indebted to R. B. Corey for suggesting a leak of this simple construction.

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DURATION OF DEMONSTRABLE ANTIBODIES IN THE SERUM OF RABBITS IMMUNIZED WITH HEAT-KILLED TYPE II AND TYPE III PNEUMOCOCCI

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(Received for publication, February 13, 1935)

The duration of type specific agglutinins and protective antibodies in the blood of rabbits immunized by intravenous, intraperitoneal, intramuscular, and subcutaneous injections of heat-killed type I pneumococci have been reported in a previous paper.[1] It was found that type specific antibodies persisted longest in the blood of animals which had been injected intravenously. Consequently this method of immunization was used in the following study. In order to determine whether the antibody response would vary when strains of pneumococci of varying degrees of virulence are used for the immunizing antibody, a rabbit virulent and rabbit non-virulent strain of type II and of type III pneumococcus were used. An intraperitoneal injection of 0.1 cc. of a broth culture of the stock strain of pneumococcus type II (SAv) is necessary to kill rabbits. It was rendered rabbit virulent by rapid passage. This virulent strain, called pneumococcus II (Sv), regularly kills rabbits following intraperitoneal injection of .000001 cc. Certain strains of type III pneumococci are avirulent for rabbits and the virulence of these strains cannot be appreciably increased by rabbit passage. Other strains, however, are encountered which initially are slightly virulent for rabbits, and the virulence of these strains may be further increased by repeated passage through these animals. The rabbit avirulent strain of type III pneumococcus is designated III (SAv) and the virulent strain pneumococcus III (Sv).

In the present paper observations are reported on the length of time during which type specific antibodies are present in detectable amounts

1. J. Exper. Med. 51: 721, 1930.

in the circulation of rabbits immunized by intravenous injections of varying amounts of heat-killed (1) pneumococcus type II (SAv), (2)

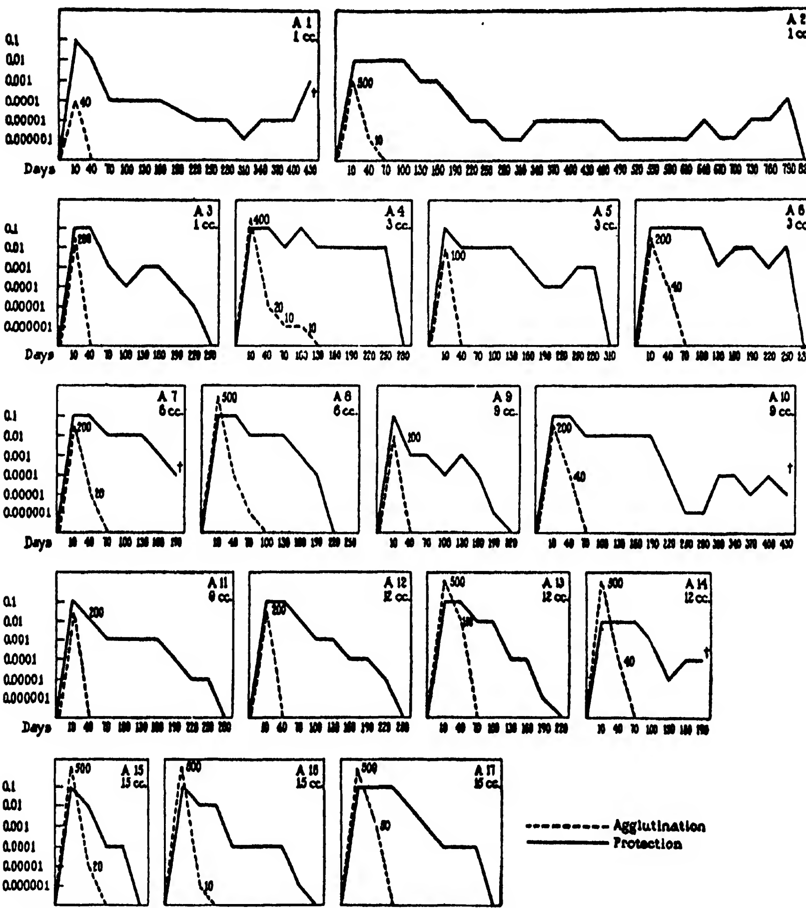

FIG. 1.—Length of time during which agglutinins and protective antibodies could be demonstrated in serum of rabbits which had been immunized intravenously with heat-killed avirulent type II (SAv) pneumococci.

pneumococcus type II (Sv), (3) pneumococcus type III (SAv), and (4) pneumococcus type III (Sv).

The methods employed were identical with those used in the previous study. Test bleedings were made 10 days after the last immun-

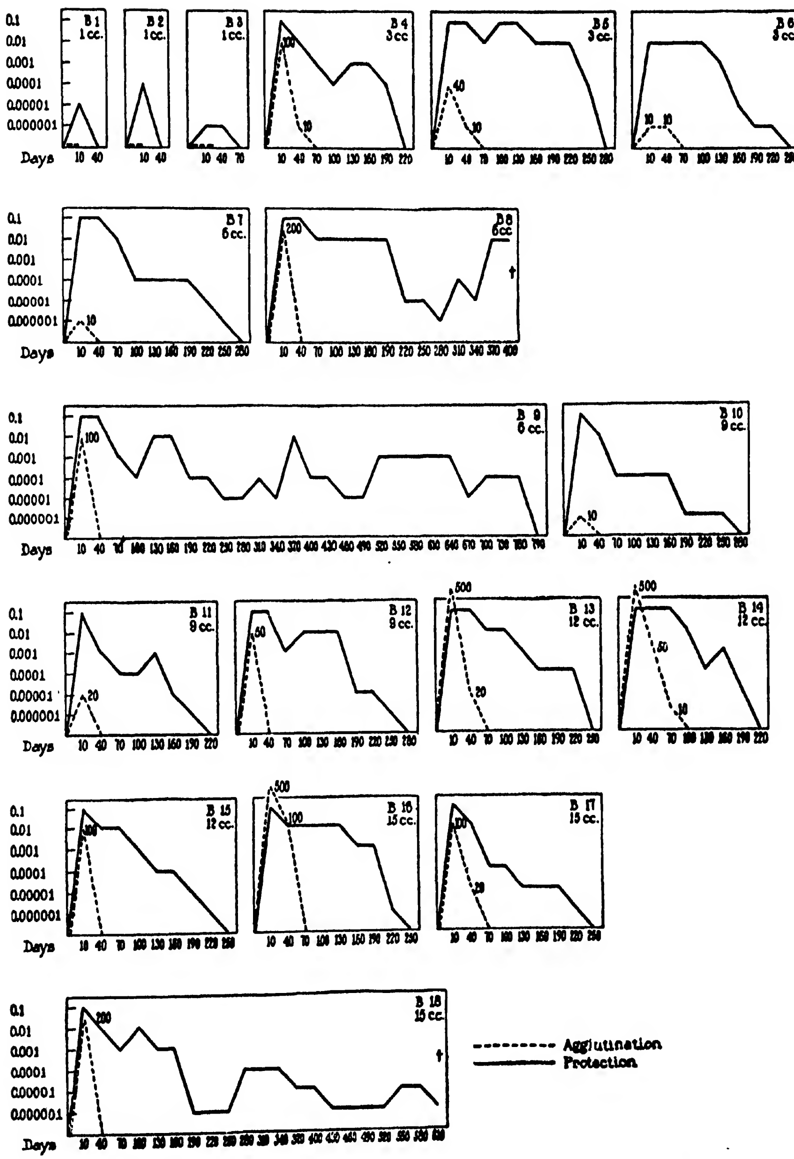

Fig. 2.—Length of time during which agglutinins and protective antibodies could be demonstrated in serum of rabbits which had been immunized intravenously with heat-killed virulent type II (Sv) pneumococci.

izing injection, and thereafter at intervals of 30 days. The antibody response and the persistence of immune bodies in the blood of the several groups of rabbits are shown in figures 1 to 4.

Rabbits Immunized with a Rabbit-Avirulent Pneumococcus Type II (SAv)

In figure 1 is shown the period of duration of antibodies in the serum of 18 rabbits which received, during the course of treatment, from 1 to 15 cc. of the suspension of heat-killed organisms. Four of the rabbits died from various causes before the observations were completed.

The serums of all these animals contained demonstrable type specific agglutinins at the time of the first test bleeding 10 days after the last injection of antigen. As a rule, however, the agglutinins lasted for relatively short periods. In 40 days they were still present in much lower concentrations in the blood of 10 of the animals, and were demonstrable after 70 days in the serum of only one rabbit.

Ten days after the course of immunization the serums of all of the animals of this group showed well marked protective power. It is remarkable that the serums of the rabbits which received a total of only 1 cc. of the bacterial suspension showed as high a titer of protective antibodies as did the serums of animals which received as much as 15 cc. of antigen. Even the duration of the protective substances seemed to bear no relation to the amount of antigen administered. Rabbit A2 which received a total of only 1 cc. of heat-killed organisms, had demonstrable antibodies in the blood when tested 790 days after the last immunizing injection. As a rule, however, the presence of protective substances was no longer detectable in the blood withdrawn from rabbits 250 days after the last injection. The series of animals is so small, however, that too much importance should not be attached to these differences.

Rabbits Immunized with a Rabbit Virulent Pneumococcus Type II (Sv)

The results obtained in the rabbits immunized by intravenous injection of similar suspensions of a rabbit virulent type II (Sv) pneumococci are shown in figure 2. All but 2 of these animals lived until demonstrable antibodies had disappeared from their blood. The 3

rabbits which received only 1 cc. of antigen failed to produce type specific agglutinins. Of the remaining animals, in only one were type specific agglutinins present in the circulation blood 40 days after the last injection of antigen. All 18 rabbits showed some protective substances in their blood 10 days after the last injection. With the exception of the 3 animals which received 1 cc. of bacterial suspension the total amount of antigen administered did not appear to bear any relation to the persistence of protective substances in the blood. Although the serum of two rabbits (B-9 and B-18) tested 760 days and 610 days respectively after immunization afforded protection to mice, as a rule the protective substances were no longer demonstrable in the blood withdrawn from rabbits approximately 250 days after immunization.

Rabbits Immunized with Type III Pneumococcus

In the past, investigators have experienced difficulty in obtaining type specific antibodies in the serum of rabbits immunized with type III pneumococcus. The literature has been well reviewed by Tillett.[2] Some workers[3] attributed their failure to variations in the original virulence of the particular type III pneumococcus employed as antigen and have concluded that rabbit avirulent strains fail to produce type specific antibodies whereas strains of rabbit virulent type III pneumococci are more effective antigenically. Tillett suggests, however, that normal rabbits possess a mechanism whereby type III pneumococci, following intravenous injection, are disintegrated in such a manner that the antigenic complex is destroyed. This may well occur in some rabbits. It is a recognized fact that individual rabbits vary in their response to immunization with pneumococcus type III. Some rabbits will produce type specific antibodies whereas others fail to do so.

In the course of investigations on the duration of antibodies in the blood of rabbits following injection of type III pneumococci, marked variations have been encountered in the response of individual rabbits. In table 1 are shown the development of type specific agglutinins and protective antibodies in the serums of 64 rabbits which had received

2. J. Exper. Med. **45:** 713, 1927.
3. Yoshioha, M.: Ztschr. f. Hyg. u. Infektionskr. **97:** 408, 1923.

TABLE 1

No. of Inoculations	Total Amount of Inoculum	Agglutinins and Protective Antibodies					
		Rabbit Avirulent Pneumococcus III Av			Rabbit Virulent Pneumococcus III Sv		
		Type Spec. Agglutinins		Amount of Culture Against which 0.5 cc. of Serum Protected Mice	Type Spec. Agglutinins		Amount of Culture Against which 0.5 cc. of Serum Protected Mice
2	1 cc.	C 1	..	..	D 1	..	..
		2	..	..	2	..	..
		3	..	..	3	..	..
		4	..	.000001	4	..	..
		5	.	.00001	5	..	..
		6	..	.00001	6	..	..
		7	..	.001	7	..	..
		8	..	..	8	..	..
		9	..	..	9	..	..
		10	..	..	10	..	..
		11	..	..			
2	3 cc.	12	..	.0001	11	..	.01
		13	..	.001	12	..	..
		14	1–50	.01	13	..	..
					14	..	..
		15	1–40	.0001	15	..	..
		16	..	.001	16	..	.0001
		17	1–40	.001	17	..	.00001
		18	..	..	18	..	..
		19	..	..	19	..	..
					20	..	..
		20	..	..	21	..	..
		21	..	.000001			
6	6 cc.	22	..	.0001	22	..	.000001
		23	..	.000001	23	..	.01
		24	1–50	.01	24	..	.001
		25	1–50	.01	25	1–50	.001
		26	1–100	.01	26	..	.0001
		27	1–100	.001	27	..	..
		28	..	.00001	28	..	..
		29	..	.00001	29	..	.000001
		30	..	..	30	..	.000001
		31	..	..	31	..	..
		32	..	.000001			

TABLE 1—*Continued*

No. of Inoculations	Total Amount of Inoculum	Agglutinins and Protective Antibodies					
		Rabbit Avirulent Pneumococcus III Av			Rabbit Virulent Pneumococcus III Sv		
		Type Spec. Agglutinins		Amount of Culture Against which 0.5 cc. of Serum Protected Mice	Type Spec. Agglutinins		Amount of Culture Against which 0.5 cc. of Serum Protected Mice
8	9 cc.	C 33	..	.001	C 32	1–50	.0001
		34	1–20	01	33	1–100	.01
		35	1–40	.01	34	..	.01
		36	1–40	.001	35	1–100	.01
					36	1–100	.001
		37	1–100	.001	37	1–200	.001
		38	1-100	01	38	1–40	.01
		39	..	.001	39	..	..
		40	..	.001	40	..	.00001
		41	..	.01	41	..	.00001
		42	..	.001	42	1–100	.001
		43	.	01	43	..	..
10	12 cc.	44	..	.01	44	1–50	.01
		45	1–10	.001	45	1–40	.001
		46	1–40	01	46	..	..
		47	1–40	.01	47	..	..
		48	1–50	.01	48	..	..
		49	..	.001	49	..	..
		50	..	.01	50	..	..
		51	..	.01	51	1–10	.01
					52	..	.01
		52	..	.0001	53	1–50	.001
					54	..	.00001
					55	..	.00001
					56	..	..
					57	..	..

TABLE 1—*Concluded*

No. of Inoculations	Total Amount of Inoculum	Agglutinins and Protective Antibodies					
		Rabbit Avirulent Pneumococcus III Av			Rabbit Virulent Pneumococcus III Sv		
		Type Spec. Agglutinins		Amount of Culture Against which 0.5 cc. of Serum Protected Mice	Type Spec. Agglutinins		Amount of Culture Against which 0.5 cc. of Serum Protected Mice
12	15 cc.	53	1-40	.01	58	..	..
		54	..	.01	59	1-40	.01
		55	1-100	.001	60	..	..
		56	1-100	.001	61	..	..
		57	1-20	.001	62	..	.000001
		58	1-100	.01	63	..	.00001
		59	..	.00001	64	..	..
		60	..	.01	65	1-10	..
		61	..	..	66	1-20	.01
		62	1-100	.01	67	1-40	.01
		63	1-40	.01	68	1-40	.01
		64	1-10	.001	69	..	.01
					70	1-40	.01
					71	1-10	.001
					72	..	.01
					73	..	.0001
					74	..	.01
					75	..	..
					76	..	..
					77	..	..
					78	..	..
					79	..	..
					80	1-20	.001
					81	1-10	.001

from 1 to 15 cc. of heat-killed avirulent type III pneumococci and of 81 rabbits which had received corresponding amounts of antigen containing the bacterial cells from a virulent culture of type III pneumococcus. Inspection of table 1 illustrated the great variations in antibody response. Although the serums of 2 rabbits receiving a total of only 3 cc. of heat-killed pneumococci type III (Av) contained demonstrable agglutinins, yet the serums of 4 other rabbits which had received 15 cc. of the same antigen failed to show agglutinins.

The variation in antibody response of rabbits to injection of heat-

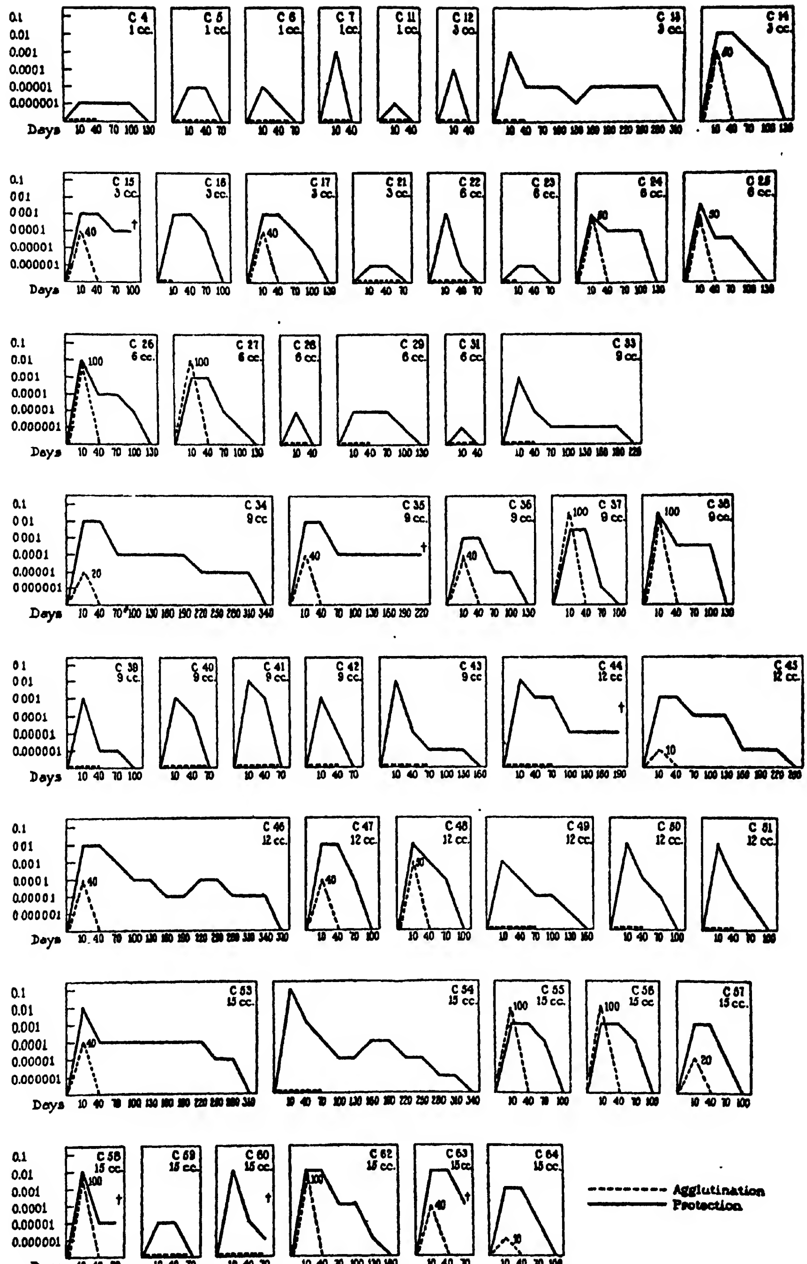

FIG. 3.—Length of time during which agglutinins and protective antibodies could be demonstrated in serum of rabbits which had been immunized intravenously with heat-killed avirulent type III (SAv) pneumococci.

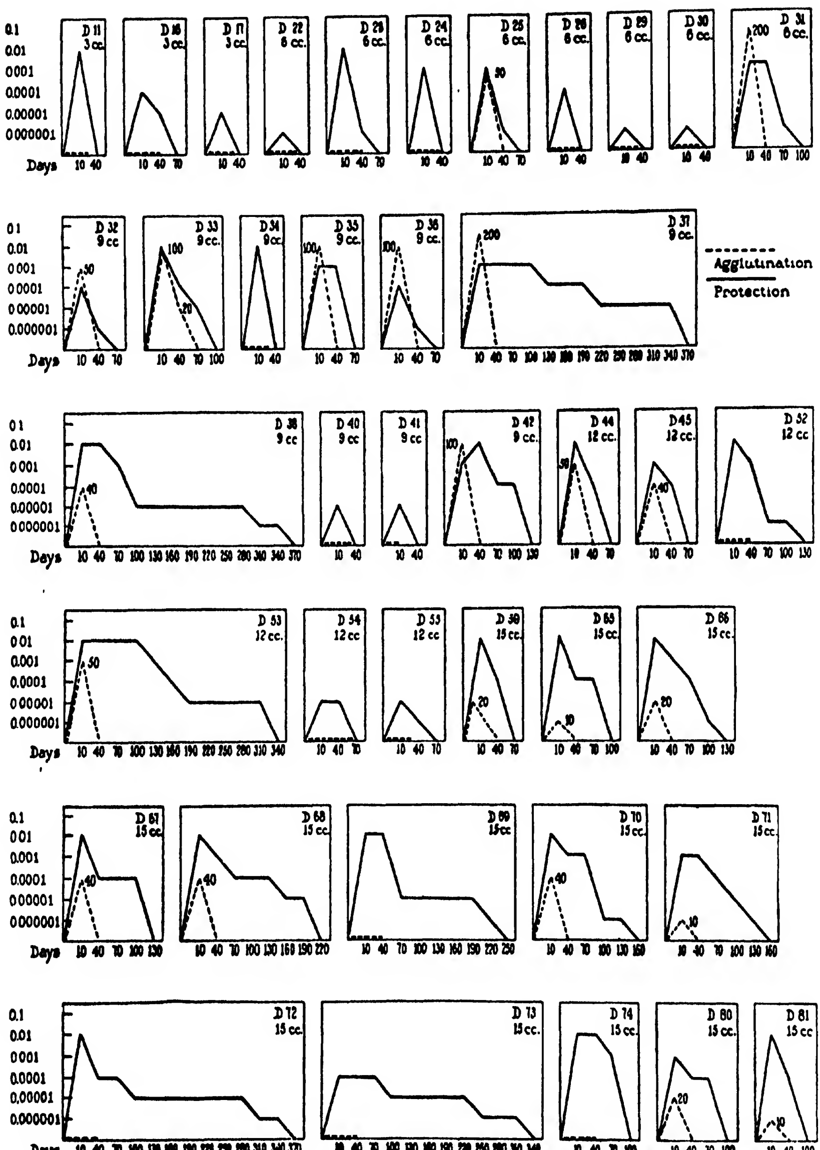

FIG. 4.—Length of time during which agglutinins and protective antibodies could be demonstrated in serum of rabbits which had been immunized intravenously with heat-killed virulent type III (Sv) pneumococci.

killed rabbit virulent pneumococcus type III (Sv) is even more marked. Whereas of the 12 animals which had received only 9 cc. of antigen, the serums of 7 or 58 per cent showed agglutinins, and 10 or 83 per cent protected mice; of the serums of 24 rabbits which had received a total of 15 cc. of heat-killed organisms only 9 or 37 per cent contained agglutinins, and 11 or 58 per cent protective antibodies. From these results it is evident that under the conditions of this experiment the production of antibodies does not depend so much on the particular strain or amount of organism injected, as the ability of the individual rabbit to disassociate or modify the antigenic complex. These experiments do not indicate the factors that may cause the destruction of the antigen.

Rabbits Immunized with a Strain of Rabbit Avirulent Pneumococcus Type III (SAv)

In figure 3 is shown the length of time type specific antibodies persisted in the serums of 64 rabbits which had received varying amounts of suspensions of a heat-killed rabbit avirulent suspension of pneumococcus type III (Av). Six of these animals died before completion of the observations. Type specific agglutinins were present in 64 or 37 per cent of these rabbits 10 days following the course of immunization. Irrespective of the initial titer of the agglutinins at this time, however, these antibodies could no longer be demonstrated in the blood withdrawn 40 days after the last immunizing injection. Even protective antibodies did not persist for long periods. In 35 or over 50 per cent of the animals these had disappeared from the circulation of treated rabbits after 130 days. In only 3 instances was blood obtained 300 days after the last injection capable of conferring passive protection on mice injected with organisms of the homologous type. The total amount of antigen injected did not seem to bear any consistent relation to the initial titer or to the duration of type specific antibodies in the circulation blood of the immunized rabbits.

Rabbits Immunized with a Strain of Rabbit Virulent Pneumococcus Type III (Sv)

In figure 4 is shown the length of time during which antibodies were demonstrable in the serum of 40 rabbits following immunization with a

rabbit virulent strain of pneumococcus type III (Sv). The blood of 21 of these animals contained demonstrable type specific agglutinins 10 days after the last injection. In all but one instance this antigen was no longer demonstrable in the circulation of rabbits after 40 days following the completion of immunization. Even the protective antibodies lasted but a short time. In only 7 instances was mouse protection obtained in 130 days after immunization. Even if the serum originally protected against 1–100 of virulent culture, this power usually disappeared rapidly.

DISCUSSION

Since individual rabbits exhibit such tremendous variations in their ability to produce type specific antibodies following injections of heat-killed suspensions of pneumococci, conclusive results can only be obtained by using large numbers of animals. It has already been shown[1] that type specific agglutinins were present only in the circulating blood for relatively short periods following intravenous immunization of rabbits with heat-killed type I pneumococci. In the present study comparable results were obtained following injection of type II and type III pneumococci.

Just as in the case of rabbits immunized with type I antigen, the serum of animals which had received 1 cc. of a bacterial suspension of type II or type III pneumococci in certain instances showed as high a titer of protective antibodies as did the serums of those which received as much as 15 cc. Although in rabbits which received the larger amounts of type I antigen the protective substances persisted longer than they did in the animals injected with smaller amounts, this phenomenon was not so apparent in the rabbits immunized with type II or type III antigen.

Despite the great individual variation in response of different rabbits to injections of heat-killed pneumococci, certain general tendencies are apparent. The total amount of inoculum bears only a slight relation to the titer of type specific agglutinins and has little or no relation to the titer or duration of protective antibodies. With all three of the types of pneumococci employed agglutinins were demonstrable in the serum of the tested rabbits for only short periods of time. Protective antibodies, however, persisted longest in the circulating blood of rab-

bits following immunization with type I organisms. They disappeared sooner from the serum of type II rabbits, and lasted the shortest time in the blood of type III animals.

The results of the present experiments show that an antigen prepared from a rabbit's non-virulent culture of either pneumococcus type II or type III stimulated antibody production in rabbits as effectively as did an antigen made from rabbit virulent organisms of these types.

CONCLUSIONS

Development of type specific agglutinins and protective antibodies bears little or no relation to the amounts of antigen administered.

Comparative analysis shows that type specific agglutinins persist for relatively short periods in the circulating blood.

Type specific protective antibodies persist longest following immunization with type I pneumococci, disappear sooner following injections of type II organisms, and last but a short period following treatment with type III antigen.

Antibody response does not appear to bear any relation to the virulence of the culture used as antigen.

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THE REACTION OF PARTIALLY IMMUNIZED RABBITS TO INHALATION OF TYPE I PNEUMOCOCCI

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Pulmonary lesions in which the tissue changes resemble those seen in acute lobar pneumonia in man have been produced in previously immunized mice which, during the initial period of alcoholic narcosis were exposed to inhalation of virulent pneumococci.[1] Furthermore, the production of these lesions is not dependent upon a local sensitization of the lung but is associated with a general immunity of a mild degree.[2]

In the present paper are reported (1) the mortality, and (2) the pulmonary changes in rabbits which had previously received varying amounts of a suspension of heat-killed type I pneumococci and were subsequently exposed to inhalations of live organisms of a homologous type. For purposes of comparison, certain of the immunized animals, together with normal controls were subjected to the influence of alcohol just prior to exposure.

EXPERIMENTAL

The antigen used to stimulate partial immunity in the rabbits consisted of heat-killed type I pneumococci suspended in salt solution. One cc. of the suspension was equivalent in bacterial content to 10 cc. of an 18 hour broth culture. Different groups of rabbits, received a single intravenous injection of from .000001 to .5 cc. of bacterial suspension. Ten days after the immunizing injection, the rabbits were placed in the spray chamber previously described[3] and were sprayed

1. Stillman, E. G., and Branch, A.: J. Exper. Med. 40: 733 and 743, 1924.
2. Stillman, E. G., and Branch, A.: J. Exper. Med. 54: 623, 1931.
3. Stillman, E. G.: J. Exper. Med. 38: 117, 1923.

with 150 cc. of an actively growing broth culture of pneumococcus type I. An intraperitoneal injection of .000001 cc. of a broth culture of the strain of pneumococcus used invariably proved fatal to normal rabbits. Alcohol was administered to one series of animals by intraperitoneal injection of a 20 per cent solution in normal saline. It was found that body weight was no criterion of the amount of alcohol an individual rabbit would tolerate. Sufficient alcohol was administered to each animal to produce deep intoxication as evidenced by lack of co-ordination of hind legs upon exertion. Heart blood cultures were plated on blood agar for further identification. Lung tissues were fixed in Zenker's fluid, embedded in paraffin and sections stained with eosin-methylene blue. All sprayed rabbits were kept under observation for a period of 14 days following exposure.

TABLE 1

Mortality of Partially Immunized Rabbits Following Inhalation of Type I Pneumococci

	Amount of Suspension in cc. used for Immunization		
	0.1–.5	.00001–.001	Normal Control
No. of rabbits exposed	42	41	16
No. died	4	18	9
Mortality, per cent	9	44	56

Mortality of Partially Immunized Rabbits

The mortality of the 83 rabbits which had been previously immunized by a single intravenous injection of heat-killed organisms, and of 16 normal animals which were exposed to a spray of living pneumococci are shown in table 1. Of the 42 rabbits which had previously received a single immunizing injection of from 0.01 to 0.5 cc. of a suspension of heat-killed pneumococci, only 4 or 9 per cent died of pneumococcus septicemia, whereas 18 or 44 per cent of the 41 rabbits which had been partially immunized by the injection of from .000001 to .001 cc. of the same antigen died. Of the 16 normal control rabbits which were sprayed, 9 or 56 per cent died of pneumococcus septicemia.

The mortality rate shown in table 1 indicates that the range of effective protection was greatest in those rabbits which had received .01

cc. or more of antigen. In this series the mortality was less than 10 per cent. In the second series, in which the rabbits received .001 cc. or less of antigen, the mortality rose to 44 per cent. Although the number of bacteria gaining entrance to the respiratory tract following spraying cannot be controlled and the degree of immunity resulting from a single inoculation of antigen varies in individual cases, there are striking differences in the two series of animals. The fact that 56 per cent of normal rabbits died following exposure to infection by inhalation, indicates that the animals which had been injected with the smaller amounts of antigen possessed some resistance.

Gross and Microscopic Pathology of Partially Immunized Rabbits

Serofibrinous pleurisy and pericarditis were often present although no evidence of consolidation of the lungs was found. Histological examinations were made of the lungs of 18 of the 22 partially immunized rabbits which died. In 9 instances the lungs were essentially normal in that there was no appreciable congestion, edema, deposit of fibrin, or cellular infiltration. In 6, the lungs were congested and in 2 cases there was in addition to congestion, a slight deposit of fibrin in the alveoli and lymphatics. In one instance, there was an extensive inflammatory reaction, which involved chiefly the interstitial tissue about blood vessels and bronchi and appeared to extend secondarily to the adjacent alveoli. The area of inflammatory cell infiltration appeared as a collar-like zone about the vessels and bronchi, involving the larger as well as the smaller branches. It was first seen in the peribronchial and perilymphatics of these areas. Massive consolidation, as seen in lobar pneumonia, was not found.

Previous studies in mice suggest that the pulmonary lesions produced denote an attempt on the part of the host to localize the infection. Although serofibrinous pleurisy and pericarditis were often present in rabbits, gross and microscopic examination failed to indicate any attempt, except in this one instance, on the part of the animal to localize the infection. Although little importance can be attached to the lesions in a single animal, nevertheless this inflammatory reaction was of interest in the light of more recently observed histological changes in rabbits infected by intranasal instillation. The variations in the histological appearance of the lung apparently bore no relation

to the amount of antigen which the animal had received. This is in striking contrast to the mortality rate as has been previously pointed out.

Effect of Alcoholic Intoxication upon the Mortality of Partially Immunized Rabbits Exposed to Inhalation of Living Pneumococci

The effect of alcoholic intoxication upon the mortality of 36 partially immunized rabbits and of 5 normal control animals is shown in table 2. From this table it is seen that the mortality of the alcoholized rabbits was approximately the same as that of the non-alcoholized animals in the previous experiment. Of the 18 animals which had previously been immunized by a single injection of 0.01 to 0.5 cc. of

TABLE 2

Effect of Alcoholic Intoxication upon the Mortality of Partially Immunized Rabbits Following Inhalation of Type I Pneumococci while Alcoholized

	Amount of Suspension in cc. used for Immunization		
	.01–.5	.00001–.001	Normal Control
No. of rabbits exposed	18	18	5
No. died	1	8	4
Mortality, per cent	5	44	80

antigen only 1 or 5 per cent died, whereas 8 or 44 per cent of the 18 rabbits which had been immunized by the injection of from .000001 to .001 cc. died. Four or 80 per cent of the 5 control animals died.

Gross and Microscopic Examination

Serofibrinous pleurisy and pericarditis were likewise present in many of the alcoholized animals. Consolidation of the lungs was not present in any of the animals. Histological examination revealed congestion of the blood vessels, at times serum precipitate was present in the alveoli, and occasionally a slight deposit of fibrin. Some of the perivascular and peribronchial lymphatics were dilated and in a few there was a small amount of fibrin. Purulent exudate was not seen. The histological changes did not suggest an attempt on the part of the rabbits to localize the infection. The histological changes in the lungs of control rabbits were similar to those observed in the non-intoxicated animals.

DISCUSSION

To Thalamon[4] is given the credit for first producing pulmonary lesions in rabbits by the intrathoracic method. Gamaleia[5] states that, in order to produce pulmonary lesions, since a virulent pneumococcus rapidly kills susceptible animals with an overwhelming septicemia without any pulmonary localization, it is necessary to use an attenuated strain of pneumococcus. Wadsworth[6] expressed the belief that there exists a subtle equilibrium between the resistance of the animal and the virulence of the micro-organism. He found that normal rabbits died of a fulminating septicemia without pulmonary lesions following intratracheal injections of highly virulent pneumococci. On the other hand, animals which had acquired a certain degree of immunity developed pneumonia under the same conditions.

All of the "successful results" of experimental production of lobar pneumonia reported in rabbits, without discriminating between those of doubtful and actual value, have been obtained by the method of intrathoracic or intratracheal inoculation. The present method, that of inhalation, is generally admitted to have failed. Selter[7] using a strain which had been isolated from an epidemic of pneumonia in rabbits, was unable to produce experimental pneumonia in normal animals exposed in a box to a spray of pneumococci.

In the present studies, the inhalation method was employed because it is less artificial and removes the objection of the mechanical injury resulting from the introduction of the catheter. In addition, the lungs of 166 rabbits which died of a pneumococcus septicemia following spraying with different types of pneumococci in other experiments have also been examined. As a number of these animals had been repeatedly exposed to inhalation of pneumococci, some degree of active immunity must have developed before infection occurred.[8] Although serous and serofibrinous pleurisy and serofibrinous pericarditis were common, in no instance was there any gross evidence of

4. Progrès méd. **11:** 281 and 301, 1883.
5. Ann. Inst. Pasteur **2:** 440, 1888.
6. Am. J. Med. Sc. **127:** 851, 1904.
7. Ztschr. f. Hyg. u. Infektionskr. **54:** 347, 1906.
8. Stillman, E. G., and Branch, A.: J. Exper. Med. **44:** 581, 1926; Stillman, E. G.: J. Exper. Med. **52:** 215, 1930.

pneumonia. In the first group of the present experiment, histological examination of the lungs of 18 of the partially immunized rabbits which died following inhalations of type I pneumococci, sections of the lungs of only one animal showed any histological evidence of an at tempt at localization of the infection. Furthermore, the histological examination of the lungs of 9 of the partially immunized alcohol intoxicated rabbits reported in the present study showed no evidence of attempts on the part of rabbits to confine the pneumococcus infection in the lungs. From these experiments it would seem that pneumococcus lobar pneumonia cannot be produced, even in partially resistant rabbits by the inhalation method.

A comparison of the mortality in the two series reveals no essential difference. In both groups the mortality was low in the animals which had received more than 0.01 cc. of heat-killed organisms and high in those receiving less. There, however, was some gradation in the mortality rate of the animals tending to assume an inverse ratio to the amount of antigen injected. Little importance can be given to the slightly higher mortality in the alcoholized control series. No essential microscopic difference existed in the pulmonary lesions of the sober or alcoholized animals.

In contrast to the results obtained in rabbits, it has been shown that in partially immunized mice which have been exposed to a pneumococcus spray while alcoholized, localization of the infection and true lobar pneumonia not infrequently develop. From these results it is clear that by the inhalation method pulmonary localization may be produced in suitable animals.

Significant observations have been made on the differences in the lymphatic anatomy of the lungs of rabbits and guinea pigs in the study of experimental tuberculosis in these animals.[9] The guinea pig has what may be called "open" lungs. Small numbers of bacilli coming into such lungs will be carried, for the most part, through them to the adjacent lymph nodes. In the "closed" lungs of the rabbits, however, there are innumerable masses of lymphoid tissue which are much more highly developed than those in guinea pig lungs. These masses are in such locations that they may intercept the progress of the bacteria through the lymphatic system. Following experimental inocu-

9. Krause, A. K.: The Harvey Lectures, 1922.

lation of the tubercle bacilli, there is a marked tendency for these organisms to accumulate in the depths of the lungs of rabbits, but following similar inoculation of guinea pigs, the bacilli show little tendency to localize in the lungs but pass through to the lymph nodes. Unfortunately no anatomical study of the lungs of mice is available. The different pulmonary reactions induced in mice and rabbits following inhalation of pneumococci may be due to the lymphatic anatomy of the lungs of these two species.

CONCLUSIONS

Following inhalation of virulent pneumococci, partially immunized rabbits failed to show any evidence of pulmonary localization of the infection.

An increased resistance to virulent pneumococci may be established in rabbits by a single injection of a small amount of antigen.

Alcohol intoxication of partially immunized rabbits did not materially alter the mortality or pulmonary reactions following infection by inhalation.

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THE REACTION OF NORMAL AND PARTIALLY IMMUNIZED RABBITS TO INTRANASAL INSTILLATION OF TYPE I PNEUMOCOCCI

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It has been shown that in partially immunized mice which have been exposed to a pneumococcus spray while alcoholized, true lobar pneumonia not infrequently develops.[1] Following inhalations of virulent pneumococci, however, partially immunized rabbits failed to show any evidence of pulmonary localization of the infection.

Since by the spray method the number of organisms entering the body is never great, and the dissemination of organisms is uncertain, the method of intranasal instillation was used in the present study. Bull[2] has shown that rabbits may be readily infected by this route. By this technique the number of organisms implanted on the mucosa of the upper respiratory tract can be more accurately controlled than by the spray method previously employed.

In the present paper the following experimental data are reported— (1) the distribution of pneumococci in the organs of normal rabbits following intranasal instillation, (2) the mortality of normal and alcoholized rabbits following intranasal instillation of varying amounts of a rabbit-virulent strain of type I pneumococci, (3) the mortality of partially immunized normal and alcoholized rabbits following intranasal instillation of a constant amount of a rabbit virulent culture of type I pneumococci, and (4) the resultant pulmonary changes.

Experimental Method

Intranasal instillation was the method employed in infecting the rabbits with type I pneumococci. A single instillation was made in

1. Stillman, E. G., and Schulz, R. Z.: *J. Inf. Dis.* **53**: *233, 1935.*
2. Bull, C. B., and McKee, C. M.: Am. J. Hyg. **7**: 627, 1927.

one nostril, care being taken not to injure the nasal mucosa. The culture of type I pneumococcus used was of such virulence as to cause the death of rabbits when doses of 0.000001 cc. of an 18 hour broth culture were given intraperitoneally. Four series of rabbits were used. In the first two series, consisting of normal and normal alcoholized rabbits, the amount of infecting inoculum instilled varied from 0.000001 to 1 cc. of an 18 hour broth culture. In the third and fourth series composed of partially immunized rabbits, 1 cc. of broth culture was introduced into the nose. Alcohol intoxication was produced by intraperitoneal injection of varying amounts of a 20 per cent solution of alcohol in physiological salt solution. As the tolerance for alcohol varied in different animals, a sufficient quantity was given to produce a loss of co-ordination of the hind legs.

The antigen used to stimulate partial immunity in certain rabbits consisted of heat-killed type I pneumococci suspended in salt solution. One cc. of the suspension was equivalent in bacterial content to 10 cc. of an 18 hour broth culture. Different groups of rabbits received a single intravenous injection of from .000001 to .5 cc. of antigen. The pneumococcus culture was introduced into the nose 10 days after the immunizing injection. In the case of the rabbits which were sacrificed at varying intervals following exposure, cultures in broth were made of blood taken from the right and left ventricles, pieces of spleen, kidney and peripheral portions of the lungs. Cultures of heart's blood alone were made of the animals which died. All positive cultures were plated on blood agar for further identification of the organism. Blocks of lung tissue were fixed in Zenker's fluid, embedded in paraffin, and sections stained with eosin and methylene blue and gram stain. All animals which did not die or were not sacrificed, were kept under observation for a period of fourteen days following inoculation.

Distribution of Pneumococci in the Organs of Normal Rabbits after Intranasal Instillation of Pneumococci

In figure 1 is shown the distribution of pneumococci in the organs of 36 normal rabbits which were sacrificed at varying intervals after the instillation of 1 cc. of a virulent culture of type I pneumococci. It will be seen from the data presented that from the 24 rabbits killed within 6 hours after nasal instillation, pneumococci were frequently

recovered from the periphery of the lungs. In three instances, pneumococci were isolated from one or two organs other than the lungs. It is interesting to observe that cultures of the blood made at this time remained sterile in all but one case. In the animals which were sacrificed 24 hours or later, the occurrence of positive cultures from the various organs runs parallel to the incidence of positive cultures from the heart's blood.

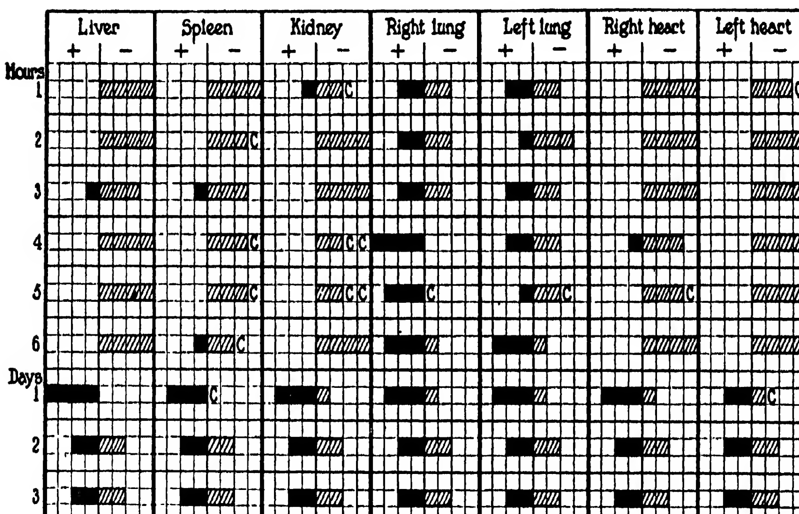

FIG. 1.—Distribution of pneumococci in organs of normal rabbits following nasal instillation of 1 cc. of a virulent culture of pneumococcus type I. The black squares indicate organs in which cultures showed pneumococci to be present, the cross-hatched squares represent organs in which cultures remained sterile. C denotes contamination.

From these results, it is evident that following intranasal instillation of 1 cc. of a virulent culture, pneumococci rapidly reach the periphery of the lungs. The occurrence of positive cultures from the viscera at a time when the blood remained sterile indicates that a transient bacteremia may have occurred. In over 50 per cent of the animals sacrificed later, the positive cultures denote a terminal septicemia.

Mortality of Normal Rabbits Following Intranasal Instillation of Virulent Culture of Type I Pneumococcus

In table 1 is shown the mortality of 75 normal rabbits following intranasal injection of from 1 cc. to .000001 cc. of a virulent culture of type I pneumococcus. All rabbits which received 1 cc. of culture died of pneumococcus septicemia. Five or 55 per cent of those which received .1 cc. died, whereas all the rabbits which had received .0001 cc. or less of culture survived. Under the conditions of this experiment the mortality of rabbits varied with relation to the number of organisms instilled into the nasal cavity.

TABLE 1

Mortality of Normal Rabbits Following Intranasal Instillation of from .000001 to 1 Cc. of Virulent Cultures of Type I Pneumococcus

Amount of Culture in cc.	1	.5	.1	.01	.001	.0001	.00001	.000001
No. of rabbits	10	10	9	9	9	10	10	8
No. died	10	6	5	1	2	0	0	0
Per cent mortality	100	60	55	11	22	0	0	0

TABLE 2

Effect of Alcohol on Mortality of Rabbits Following Intranasal Instillation of from .000001 Cc. to 1 Cc. of Virulent Cultures of Type I Pneumococcus

Amount of Culture in cc.	1	.5	.1	.01	.001	.0001	.00001	000001
No. of rabbits	5	5	5	5	5	5	5	5
No. died	4	4	2	2	1	0	1	0
Per cent mortality	80	80	40	40	20	0	20	0

Effect of Alcohol on the Mortality of Rabbits Following Intranasal Inoculation of Virulent Cultures of Type I Pneumococci

To determine what effect the administration of alcohol might have on the susceptibility of rabbits to intranasal infection, 40 animals were given sufficient alcohol to produce deep intoxication. While under the influence of alcohol, they were infected with varying amounts of pneumococcus culture intranasally. The results of this experiment are shown in table 2. From this table it is seen that the

administration of alcohol did not appear to have rendered the animals more susceptible to infection by intranasal instillations of varying amounts of a pneumococcus culture.

Distribution of Pneumococci in the Organs of Rabbits Following Intranasal Instillation of .001 Cc. of Virulent Culture of Type I Pneumococcus

To determine whether the lower mortality associated with instillation of smaller amounts of culture into the nose of rabbits was depend-

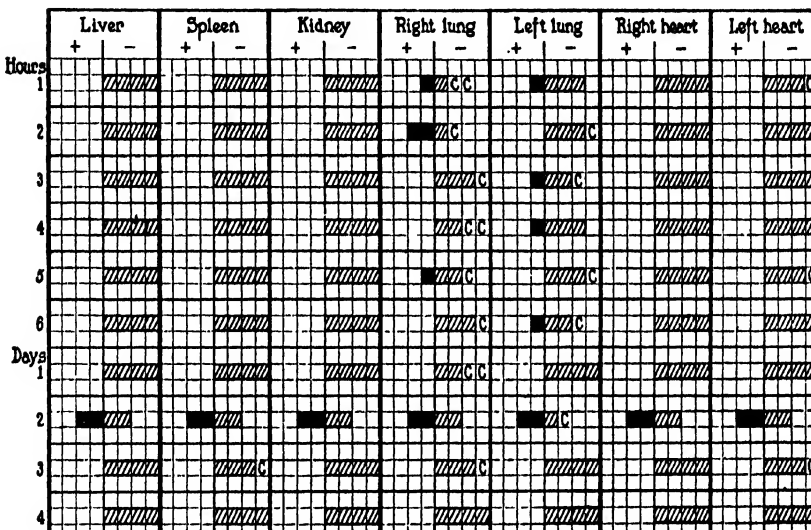

FIG. 2.—Distribution of pneumococci in organs of normal rabbits following nasal instillation of .001 cc. of virulent pneumococcus culture. The black squares indicate organs in which cultures showed pneumococci to be present, the cross-hatched squares represent organs in which cultures remained sterile. C denotes contamination.

ent on the failure of the organisms to penetrate the body, the following experiment was performed. Each of 40 rabbits received 0.001 cc. of a virulent culture of type I pneumococcus. In figure 2 is shown the distribution of pneumococci in the organs of these rabbits. From figure 2 it is seen that following instillation of a relatively small number of pneumococci into the nose, the organisms reach the periphery of the lung in certain instances. The micro-organisms, however,

rarely invade the other tissues, as shown by the absence of positive cultures of the liver, spleen, kidney or heart's blood. Whether this lack of penetration was due to the destruction or fixation of organisms in the upper respiratory tract, cannot be determined from these experiments. Microscopic studies of animals which died suggest that fixation tends to occur in the lungs of some rabbits. The negative cultures from the heart and from the parenchymal tissues of other organs, add support to the concept that little invasion of the body or dissemination had taken place.

TABLE 3

Mortality of Partially Immunized Rabbits Following Intranasal Instillation of 1 Cc. of Virulent Type I Pneumococcus Culture

Amount of Inoculum in cc. used for Immunization	.5	.25	.1	.01	.001	.0001	.00001	.000001	Normal Controls
No. of rabbits	10	10	10	10	10	10	10	10	3
No. died	0	0	2	3	5	8	7	9	3
Per cent mortality	0	0	20	33	50	80	70	90	100

TABLE 4

Mortality of Partially Immunized Alcoholized Rabbits Following Intranasal Instillation of 1 Cc. of Virulent Culture of Type I Pneumococcus

Amount of Inoculum in cc. used for Immunization	.5	.25	.1	.01	.001	.0001	00001	.000001	Normal Controls
No. of rabbits	5	5	5	5	5	5	4	5	3
No. died	0	0	0	0	3	4	3	3	3
Per cent mortality	0	0	0	0	60	80	75	60	100

Mortality of Partially Immunized Rabbits Following Intranasal Instillation of 1 Cc. of Virulent Culture of Type I Pneumococci

As the results of the previous experiments show, if a sufficient number of organisms are instilled, rabbits may be regularly infected. To determine if increased resistance would have any effect on the course of the infection, a similar series of rabbits was partially immunized by a single injection of heat-killed organisms and infected intranasally. The results of this experiment are shown in table 3. From table 3 it is seen that of the 80 rabbits which had previously been partially immunized, 34, or 42 per cent died of pneumococcus septicemia. The

10 rabbits which had previously received .25 cc. to .5 cc. of immunizing antigen survived. The mortality was higher among the rabbits which had received the smallest amount of immunizing antigen. All three non-immunized control animals died.

Effect of Alcohol on the Mortality of Partially Immunized Rabbits Following Intranasal Instillation of 1 cc. of Virulent Culture of Type I Pneumococcus

In this experiment 39 partially immunized rabbits were nasally infected during profound alcoholic intoxication. The results are presented in table 4, in which it is seen that of 39 rabbits, 13, or 33 per cent died of pneumococcus septicemia. It is also evident that the resistance of these rabbits which had been previously immunized by a single injection of antigen, in amounts greater than .01 cc. is sufficiently enhanced to protect them against injection of 1 cc. of virulent culture. On the other hand, 60 80 per cent of the animals which had received less than .001 cc. of heat-killed organisms were fatally infected. As all 3 control rabbits died, the preliminary immunization would appear to have induced some degree of resistance.

Pulmonary Lesions Following Intranasal Instillation of Type I Pneumococci

If any evidence of pulmonary localization of pneumococcus infection could be produced in rabbits following intranasal instillation, such lesions should be present in the lungs of these rabbits. No difference could be detected, however, between the gross appearance of the lungs from normal or partially immunized animals. Although there was no gross evidence of consolidation of the lungs in any of the rabbits which died, serofibrinous pleurisy and pericarditis often occurred.

The lungs of 88 of the 91 rabbits that died following intranasal instillation were examined microscopically. Irrespective of the preceding treatment the animals may have received, the lungs of these rabbits presented similar histological changes. For the convenience of description, the changes which occurred in the lungs may be described in three types. In reality there was an overlapping of these types, as well as considerable variation in the intensity of the lesions.

The first type of lesion was seen in 12 animals, 13 per cent, which

were considered essentially normal in that there was no congestion, edema, or deposition of fibrin.

In the second group were 32 animals (33 per cent). In the lungs of these rabbits there was congestion, edema, and a deposit of varying amounts of fibrin. The edema and deposit of fibrin seemed to be present in the interstitial tissue, and was accompanied by dilatation of lymphatics. In many instances where the changes were prominent

FIG. 3.—CK 12 43. Illustrates general distribution of exudate chiefly localized to the perivascular lymphatics. Note the practical absence of bronchial exudate. ×90.

in the interstitial tissue, and where a dilatation of the lymphatics was marked, the bronchi and alveoli were frequently only slightly involved. In comparatively few instances was this process reversed.

The lungs of the third group of 44 animals, 50 per cent, showed evidence of a definite purulent exudate. The most striking feature in the third group was the collar-like zone of polymorphonuclear infiltration about blood vessels and bronchi (figure 3). The latter were

not always completely circumscribed. This infiltration in the more massive areas completely obscured the surrounding structures. Where the infiltration was less marked it was found to be in the dilated lymphatics and in the adjacent edematous fibrous tissue. In the areas

Fig. 4

Fig. 5

Fig. 4. CK 20-43. Low power view showing general distribution of exudate. Note the slight involvement of the peribronchial zone as compared with the perivascular area. Bronchi containing chiefly serum, red blood cells and comparatively few polymorphonuclear leucocytes. ×90.

Fig. 5.—CK 20-43. Higher power of figure 2 illustrates the extensive filling of the perivascular lymphatics by fibrin and polymorphonuclear leucocytes. Note the swelling of the artery wall and the infiltration by polymorphonuclear leucocytes.

of extensive involvement there was not infrequently an infiltration of the wall of the vessels (figures 4 and 5). Where the infiltration had extended to the intima, there was beginning thrombus formation. Small blood vessels were infrequently thrombosed. In addition to the infiltration, there was a thickening of the vessel wall due to a

separation of the muscle and connective tissue fibers which at times were fragmented. The lumen of the bronchi contained varying amounts of purulent exudate. The amount, however, was usually not large. The epithelium was well preserved. There was some infiltration of the bronchial wall. This infiltration was most prominent in the outer layers, and was continuous with the process in the interstitial tissue. Alveoli in the regions of interstitial tissue involvement were frequently filled with polymorphonuclear leucocytes and fibrin. Alveoli removed from the areas of interstitial involvement were usually normal, or contained a small amount of serum precipitate. The pleura in 25 cases was covered by fibrin which contained polymorphonuclear leucocytes. Nowhere was there the massive uniform consolidation seen in lobar pneumonia.

Although the lungs of a certain number of animals showed an interstitial inflammatory process, the number of days the animal survived following intranasal instillation of the pneumococci bore no relation to the degree or type of the reaction in the lungs. In most animals which died 2 to 4 days after inoculation, extensive cellular reactions were present, but in other animals which survived 6 or 7 days, only slight pulmonary changes had occurred.

In general, in the animals which had received varying amounts of inoculum, the most constant and marked infiltration of inflammatory cells and fibrin deposit occurred in those animals which had received .001 cc. or less of the culture. In the partially immunized rabbits, the more extensive inflammatory reaction tended to occur in the animals which had the smaller amounts of antigen. The frequency and the degree of reaction in both the above named conditions was, however, insufficiently constant to warrant a definite conclusion.

DISCUSSION

From these experiments it is seen that following intranasal instillation of pneumococci, the organisms rapidly reach the periphery of the lungs and that a transient bacteremia may also occur. It is unfortunate that the point of invasion and the pathways of dissemination of the pneumococci can not be determined. As Jones[3] has shown, the great number of other organisms which inhabit the whole respiratory tract as deep as the bronchi, renders cultural studies impracticable

3. J. Exper. Med. **36**: 317, 1922.

except when made at the periphery of the lung. Histological detection of pneumococci in the tissues is also unreliable when the organisms are present only in small numbers.

These experiments throw no light on the actual sites of penetration. They do show, however, that the number of organisms which are introduced into the nose bears a direct relation to the number of animals which develop a fatal septicemia. In all probability the majority of organisms are destroyed either where first implanted on the mucous membranes, or deeper in the tissues by the endothelial leucocytes or by polymorphonuclear leucocytes. In any case, a few organisms occasionally filter through into the blood stream. Not only are there probably few organisms free at any one time in the circulating blood, but these few bacteria may even be within leucocytes and not in reality multiplying in the blood.

Alcoholic intoxication does not apparently render rabbits more susceptible to infection following intranasal inoculation.

Immunized rabbits survived an otherwise fatal instillation of pneumococci in direct proportion to the amount of antigen used for immunization. The lesions produced were first manifest in the perivascular and peribronchial lymphatics and later involved the adjacent tissues. Histologically, a lobar type of consolidation did not occur in any of the animals. In view of the fact that the organisms were introduced into the nose, the bronchi and alveoli showed surprisingly little involvement. In striking contrast to this, was the occurrence of vascular thrombosis and the infiltration of the walls of the blood vessels by polymorphonuclear leucocytes. The histology of the inflammatory reaction is practically identical in all animals; normal, and partially immunized. In all the animals which succumbed, the pathological lesions were similar, irrespective of the number of days which elapsed before death. By the method of intranasal instillation, under the conditions of this experiment, pneumococcus lobar pneumonia cannot be produced even in partially resistant rabbits by intranasal instillation. An interstitial inflammation was, however, present in 83 per cent of the animals which died.

CONCLUSIONS

Rabbits may be more regularly infected by intranasal instillation than by inhalation of pneumococci.

The mortality of rabbits so infected varies in direct proportion to the number of pneumococci that are instilled into the nose.

A single intoxicating dose of alcohol does not materially alter the mortality of rabbits following intranasal instillation.

Following intranasal inoculation, the perivascular lymphatics may be characteristically involved. The dilatation of the lumen of the lymphatics, and the infiltration of the surrounding interstitial tissue by leucocytes, often formed a definite collar about the larger vessels. In the more advanced cases, the walls of the vessels themselves are infiltrated by leucocytes, and vascular thrombi are finally formed.

Under the conditions of these experiments, lobar pneumonia could not be produced.

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IMMUNOLOGICAL AND CHEMICAL INVESTIGATIONS OF VACCINE VIRUS

III. RESPONSE OF RABBITS TO INACTIVE ELEMENTARY BODIES OF VACCINIA AND TO VIRUS-FREE EXTRACTS OF VACCINE VIRUS

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In the majority of instances human beings or lower animals that have recovered from a virus infection are free from further molestation by the same agent. This immunity may endure throughout an individual's life. Vaccinations with attenuated viruses that are still active have also been shown to induce protection. In certain cases, *e.g.*, vaccination against smallpox, such measures regularly produce a mild infection. Because of objections that have been brought against the production of disease, even though mild, by vaccination, many attempts have been made in the past to obtain serviceable amounts of protection with vaccines that cause no evidences of disease. The results of this type of work have led to differences of opinion regarding tests of complete inactivation of viruses and the antigenicity of such inactivated materials. It is not surprising that much of the work has been conducted with the virus of vaccinia. The reports which are pertinent to the present investigation will be scrutinized in some detail because of the very great practical possibilities inherent in successful methods of immunizing against the viruses.

A number of investigators (1–17) have relied on the action of heat to inactivate vaccine virus, while others (26–29) employed chemical agents such as methylene blue, bile, and chloroform. Inasmuch as our experiments deal with the antigenicity of formalin-treated vaccine virus and virus-free extracts of vaccine virus, we shall call attention only to the reports dealing with results of work in this field.

Hunt and Falk (1) allowed vaccine virus to remain in contact with 0.1 per cent formalin for 12 hours and cutaneous inoculation of the material in rabbits did not produce lesions of vaccinia. Subcutaneous injections of large amounts of the treated virus were made in rabbits and the animals treated in this manner were

found to be immune. Gordon (2) found that the immunity following injections of virus suspension rendered noninfectious by the addition of 1 per cent phenol was less than that produced by injections of heat-killed vaccine. Both kinds of treated virus were shown to be inactive by dermal inoculations in rabbits. Kraus (18) added 0.5 per cent phenol and 0.3 per cent formalin to different portions of vaccine virus and then tested them by means of application on the scarified skin of a rabbit to determine whether inactivation was complete. He found that monkeys and rabbits receiving the treated virus were partially and completely immune, respectively. Bussel and Mayzner (19) added 0.1 per cent formaldehyde to vaccine virus, and held the mixture at 37°C. for 6 weeks. Tests for the presence of active virus were not mentioned. They report that children receiving the material subcutaneously were partially immune, while those who were injected intracutaneously had unusually severe reactions to subsequent vaccinations with active virus. In rabbits, Bland (20) obtained irregular results with vaccine virus inactivated by the addition of 0.1 per cent formaldehyde or 1 per cent phenol. In guinea pigs, however, he was able regularly to induce immunity with similar materials. He tested for the presence of active virus by subcutaneous inoculations of some of the material in a guinea pig followed by 2 serial passages. Biglieri (21) was unable to demonstrate an immunity in rabbits that had received formolized (0.3 per cent) virus. However, preparations inactivated by phenol (1 per cent) or ether induced immunity. Presumably, by cutaneous inoculation in rabbits, the preparations were shown to contain no active virus. In the case of phenol and ether, a long period of time was required to accomplish inactivation. Iwanoff (22) used calf lymph to which had been added various amounts of formaldehyde. Complete inactivation of the material was demonstrated by corneal inoculations in guinea pigs. Varying degrees of immunity were obtained in rabbits and guinea pigs by injections of the treated virus. Gastinel, Reilly, and Mortier (17) added 0.2 per cent of formol to vaccine virus. Failure of an eruption to appear after inoculation of such material on the skin of a rabbit was taken to indicate inactivation of the virus. Rabbits inoculated 7 times at intervals of 5 days with the treated virus were still entirely susceptible to cutaneous inoculations with active virus, although serum taken from the animals after the injections was capable of neutralizing large amounts of virus. Hilgers (23) exposed vaccine virus to the action of 0.3 per cent formalin at 37°C. for 5 days. Such material failed to infect the cornea of a rabbit and did not produce a testicular reaction. Rabbits injected subcutaneously with the treated virus either once or several times were later tested by dermal inoculation of potent virus. In one experiment most of the animals were found resistant to inoculation of active virus; in most of the animals of other experiments the course of the vaccinal infection was only slightly modified. Hilgers considers that the virus treated in this way retains some of its antigenicity, but that its immunizing action is too irregular to be relied upon for general use.

Kramer (24) passed testicular vaccine virus through specially prepared basic filters which he had previously shown to hold back vaccine virus. He does not

mention tests for the presence of active virus in the filtrates. Rabbits were inoculated with the filtrates, and if more than 7 daily injections were made, resistance to potent virus resulted. Recently, Salaman (25) used Seitz filtrates of dermal vaccine virus and found that a partial immunity developed in rabbits as a result of several injections. He states that only the filtrates proved to be free from active virus by cutaneous inoculations of guinea pigs were used for immunization.

It is obvious that the reports of results obtained with "inactive" vaccine virus are conflicting, and in the light of our present knowledge of viruses it is doubtful whether such reports are of value to one attempting to solve the problem of the antigenicity of these agents in an inactive state. In almost all cases, emulsions of infected tissues have served as the source of the vaccine virus. In such a menstruum the virucidal action of heat and chemical agents is irregular, because protein and cellular detritus present in a tissue emulsion serve as protective substances for the virus. Furthermore, the degree of heat employed, or concentration of chemical agents added has usually been only slightly, if at all greater than the minimum requirement for inactivation of the virus as determined in other experiments. Moreover, in the instances in which tests of the treated material for the presence of active virus have been described, such tests have consisted of the inoculation of only a small portion of the emulsions in guinea pigs or rabbits, whereas for purposes of immunization large amounts of the materials were used. Such a procedure, therefore, can hardly be accepted as a conclusive demonstration of the absence of small amounts of active virus from the emulsions used for immunization. Finally, according to published reports, few or no precautions were taken to prevent the accidental transmission of vaccinia to the experimental animals from others frankly infected with the malady and housed in proximity to them. That such transmission occurs readily has been shown by previous workers (30), and may take place even when careful attempts are made to prevent it, as will appear later in the present report.

Recent work (31, 32) on the purification of the elementary bodies of vaccinia has made possible an accurate study, under properly controlled conditions, of the response to these bodies in an inactive state. It is with this problem that the present paper deals. That vaccine virus is intimately associated with these bodies is shown by the fact that suspensions of them are highly infectious (32).

Methods and Materials

Virus.—The manner in which suspensions of relatively pure elementary bodies of vaccinia were obtained for the experiments has been described in detail in a previous communication (32). At this time it is sufficient to state that such preparations contain practically nothing but elementary bodies in a state of dispersion compatible with an even and regular inactivation by heat or formalin.

Inactivation of Virus.—The elementary bodies were inactivated either by formaldehyde or heat. Experience led us to use formalin and to test for the presence of active virus in treated materials in the following manner. To large quantities of elementary body suspensions sufficient formalin was added to make the final concentration of formaldehyde 0.3 per cent. The mixture of elementary bodies and formalin was allowed to remain in a refrigerator for 10 days. Then 10 cc. were removed, and the elementary bodies after sedimentation in an angle centrifuge were resuspended in 1 cc. of Locke's solution and injected into the testicle of a rabbit. 4 days later the testicle was removed, and an emulsion was prepared, 1 cc. of which was injected into the testicle of another rabbit. 4 days later another testicular passage was made. None of the rabbits evidenced signs of a vaccinal infection and when tested for immunity to vaccinia some weeks later were found to be fully susceptible. Another 10 cc. portion of the treated elementary bodies were handled in a manner similar to that described above with the exception that the testicular passages were made at 6 day intervals instead of 4. From these rabbits also no evidence of the presence of active vaccine virus in the treated material was found. Thus 20 cc. of the formalin-treated suspension of elementary bodies were tested for the presence of active virus. In none of the experiments on immunity to be described were more than 18 cc. of this material injected into a rabbit. Consequently a greater amount was tested each time than was used for immunization of a rabbit. In some experiments the elementary bodies were concentrated and then enough formaldehyde was added to make a final concentration of 10 per cent. After standing in the ice box 10 days the material underwent a tenfold dilution before being used. Other preparations of elementary bodies were inactivated by heat. To accomplish this, the desired amount of material was sealed in large ampoules which were then immersed in boiling water for 2 hours.

Soluble Antigens.—The method by which virus-free extracts of tissues containing soluble antigens were prepared has also been described in a previous paper (32). The extracts of vaccinal infected tissue, either dermal or testicular, were freed from virus by filtration through collodion membranes[1] which had an average pore diameter of 103.0 $\mu\mu$. These filtrates were then tested for presence of active virus in the manner described above and found to contain none. They did contain the soluble antigens however, as demonstrated by means of the precipitin reaction.

[1] The membranes were prepared by Dr. J. H. Bauer of the Yellow Fever Laboratory of the International Health Division, Rockefeller Foundation.

Animals.—Healthy, full grown rabbits were used in all of the experiments.

Housing of Animals.—In order to avoid the accidental transmission of vaccinia to rabbits used in the experiments, animals were kept during the period of immunization in a separate room not previously used for work on vaccinia, and rigid precautions were taken to prevent the introduction of active virus into this room. After immunization and just prior to the inoculation with active virus for the purpose of testing their resistance, the animals were transferred to a room used for work on vaccinia.

Injections.—As a rule a 6 weeks course of injections was given, the rabbits being inoculated intraperitoneally on 2 successive days of each week with increasing amounts of material, *viz.*, 0.25, 0.50, 1.0, 2.0, 2.5, and 3.0 cc.

Controls.—To control the technic of isolation, and to test the effect of heterologous antigens, certain rabbits were given no injections, while others were inoculated with typhoid vaccine or with meat infusion broth.

Tests for the Presence of Antibodies in the Sera.—Samples of serum were collected from each rabbit at different times in order to test for the development of agglutinins, precipitins, and neutralizing antibodies. The method of conducting the agglutinin and precipitin reactions has already been described (31, 32). Neutralization tests were carried out in the following manner. A virus suspension was prepared by grinding with alundum the testicles of a rabbit inoculated 4 days previously with vaccine virus. 20 cc. of Locke's solution were added; the resulting emulsion was centrifuged at 1500 R.P.M. for 10 minutes; the supernatant liquid, designated as "undiluted virus suspension," was removed with a pipette and saved. Serial tenfold dilutions of the virus emulsion were prepared, and 0.30 cc. of each were mixed with an equal volume of the serum to be tested. After incubation at room temperature for 1 hour or longer, 0.25 cc. of each mixture were inoculated intradermally in a rabbit. In order to avoid the individual variations in susceptibility of rabbits to vaccinal infections, in so far as possible all samples of serum from one experimental animal were tested on a single rabbit at the same time. By this means, consistent results in regard to changes in the neutralizing capacity of the serum of each animal were obtained.

Tests of Resistance to Vaccinia.—After completion of a course of injections and collections of serum, the degree of immunity of each rabbit was tested by the dermal and intradermal inoculation of tenfold dilutions of vaccine virus and by the intratesticular injection of 1 cc. of a 1:1000 dilution of the active agent. 3 strains of virus were used. The first had been carried for some time in culture and was of relatively low pathogenicity for the rabbit; the second was a testicular strain, highly virulent; the third was the strain used in preparing the elementary body suspensions, and was used in the form of a suspension of active elementary bodies. These strains of virus are designated as "culture," "BH," and "C.L.," respectively.

EXPERIMENTAL

It has been reported by Craigie (33), who worked under properly controlled conditions, that agglutinins, precipitins, and complement-fixing antibodies appeared in the blood of rabbits after inoculation with elementary bodies of vaccinia inactivated by formaldehyde or heat. We have extended this work to include a study of the response of rabbits to inactive elementary bodies as evidenced by the neutralizing capacity of their sera and resistance to infection with active virus. It has also been shown (31) that extracts of vaccinal infected tissues contain soluble substances reacting specifically with antivaccinal serum. We have studied the antigenicity of such extracts after removal of active virus from them by filtration through collodion membranes.

In investigations concerning the response of animals to inoculation with noninfectious preparations of vaccine virus, it is desirable that suspensions of the active agent be as free as possible from other particulate or soluble materials in order that the action of virucidal agents may be regular and uniform. Furthermore, it is desirable that the virus in the preparations be susceptible of concentration, in order that a representative sample may be tested for activity of the treated agent. A suspension of elementary bodies of vaccinia satisfies these requirements. Soluble materials and particulate matter different from the elementary bodies can be removed by repeated washing in dilute buffer and differential centrifugation, and the virus in the final preparations can be concentrated to any desired extent by means of the angle centrifuge. Moreover, in such investigations care must be taken that rabbits do not become accidentally infected with vaccinia. Consequently, throughout this work proper isolation precautions were observed.

Experiment 1

Before undertaking extensive studies to determine the response of rabbits to injections of noninfectious formolized elementary bodies of vaccinia and to virus-free extracts of dermal vaccine virus, it seemed best to carry out one or two orientation experiments.

4 rabbits were used from which samples of serum were collected before the injections were begun. 2 were inoculated with a suspension of elementary bodies

inactivated by the addition of 0.3 per cent formaldehyde, and two received injections of an extract of dermal vaccine virus freed from active virus by filtration through collodion membranes. Inoculations were made weekly over a period of 6 weeks. 15 cc. of material were given each animal. At first, injections were made intravenously, but, after the third administration of the filtrate, one of the rabbits died in convulsions within a few minutes. The remaining rabbits, therefore, were subsequently injected intraperitoneally. Blood was taken again from each animal 1 week after the last injection, and the rabbits were tested for immunity to active virus after another week. In this experiment a suspension of active elementary bodies was used in the test of resistance.

TABLE I

Results of Inoculation of Rabbits with Formolized (0.3 Per Cent) Elementary Bodies and Virus-Free Filtrate of Dermal Vaccine Virus Extract

Rabbit No.	Serum*			Inocula	Serum*				Virus inoculation†	
	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate		Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization	Strain	Titer—intradermal
8-37	–	–	–	Formolized elementary bodies	32	2	8	10^2, 10^3, 10^4	C.L.‡	10^7
8-38	–	–	–	" "	32	4	16		C.L.	10^5
8-39	–	–	–	Dermal filtrate			Died			
8-40	–	–	–	" "	8	4	4	1, 10, 10^2	C.L.	10^6
9-10				None					C.L.	10^7

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum. Neutralization tests were performed on different rabbits and results, therefore, are irregular.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ C. L. signifies Connaught Laboratory strain of vaccine virus, propagated on scarified skin.

The results of the experiment are summarized in Table I. None of the rabbits possessed agglutinins or precipitins for vaccine virus prior to the inoculations. Afterward, these antibodies were present in all. The titer of agglutinins ranged from 1:8 to 1:32; that of precipitins from 1:2 to 1:16. Neutralization experiments were carried out only on samples of serum obtained after completion of the course of re-

peated inoculations. The pooled sera of the 2 rabbits inoculated with inactive elementary bodies neutralized 10^2 to 10^4 infectious units of virus, while the serum of the animals that received virus-free extracts neutralized from 1 to 10^2 units. Although these tests were done on different rabbits at different times and the neutralization titers are not comparable, yet it is evident that the sera were capable of neutralizing moderate amounts of active virus. It is noteworthy that the animals inoculated with inactive elementary bodies possessed more humoral antibodies than did the one that received the virus-free filtrate. When tested for resistance to vaccinia by means of intradermal inoculations of active elementary bodies, the treated and control rabbits responded in a similar manner with the exception that the former developed considerable edema at the sites of inoculations shortly after the injections were made. The edema disappeared within 48 to 72 hours, after which the lesions and the titers of the virus in all of the animals were not strikingly different.

Experiment 2

In the previous experiment it was shown that agglutinating, precipitating, and virus-neutralizing antibodies appeared in the serum of rabbits inoculated repeatedly with small doses of preparations of noninfectious elementary bodies, while resistance to infection with a strong virus was not appreciably altered. It then seemed advisable to learn whether a single inoculation of a large quantity of inactive virus would be more effective.

In order to give at one time a quantity of virus similar to that previously used in repeated doses, it was desirable to reduce the volume in which it was contained. This was done as follows: A suspension of elementary bodies was prepared in the usual manner to which 0.3 per cent of formaldehyde was added. After remaining in contact with the formaldehyde for 2 weeks the elementary bodies were sedimented in the angle centrifuge and taken up in a small amount of Locke's solution. Each 1.5 cc. of the concentrated material represented the amount of virus contained in 20 cc. of the original preparation, while the concentration of formaldehyde was greatly reduced. Samples of serum were taken from two rabbits and then each animal received 1.5 cc. of the concentrated inactive virus, 0.5 cc. intradermally and 1.0 cc. intratesticularly. In neither animal did local reactions and fever occur. 2 weeks later blood was taken again for the study of humoral antibodies, after which tests for resistance to culture and BH viruses were conducted.

The results of the above experiment are set forth in Table II. Before treatment the rabbits possessed no demonstrable humoral antibodies for vaccine virus. Afterwards, however, agglutinin and precipitin titers were 1:256 and 1:2 or zero, respectively, and the sera neutralized 1 infectious dose of virus. Control and treated rabbits responded in the same manner to inoculations of BH and culture viruses. Samples of serum collected from the treated and control

TABLE II

Results of Inoculation of Rabbits with Single Massive Injections of Formolized (0.3 Per Cent) Elementary Bodies Injected Intradermally and Intratesticularly

Rabbit No.	Serum*				Inocula	Serum*				Virus inoculation†						Serum*			
	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization		Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization	Strain	Titer—intradermal	Strain	Titer—intradermal	Temperature	Testicular reaction	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization
														°F.					
5-76	–	–	–	–	Formolized elementary bodies	256	2	2	1	C‡	10^4	BH§	10^6	105.7	+	1024	16	16	10^6
5-78	–	–	–	–	" "	256	2	–	1	C	10^5	BH	10^6	105.5	+	512	16	32	10^6
5-75	–	–	–	–	None	–	–	–	–	C	10^4	BH	10^6	105.3	+	512	2	1	10^5
5-77	–	–	–	–	"	–	–	–	–	C	10^4	BH	10^6	105.6	+	512	4	4	10^6
5-79	–	–	–	–	"	–	–	–	–	C	10^4	BH	10^6	105.4	+	1024	2	1	10^6

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ C indicates culture vaccine virus.

§ BH indicates New York City Board of Health vaccine virus, propagated in testicles of rabbits.

animals after the test for resistance to active virus neutralized 10^6 infectious doses of virus and possessed agglutinin titers of 1:512 to 1:1024 and precipitin titers of 1:1 to 1:32. It is interesting to nóte that there was a marked difference between the precipitin titers, 1:16 –1:32, of serum from previously treated rabbits and the titers, 1:1–1:4, of serum from the controls that had not had any inoculations prior to the injections of active virus. This fact seems to indicate

that some change had occurred in the rabbits as a consequence of the inoculation of inactive elementary bodies although it was not evident in the tests for resistance to infection with active virus.

From the results of this experiment it is obvious that the administration of the inactive virus in 1 large dose is no more effective than repeated injections of small doses. In fact, in view of the results obtained in subsequent experiments it appears that the former method is less effective than the latter.

Experiment 3

Since it appeared from the results of the experiments just described that the rabbits showed a certain amount of response to injections of inactive elementary bodies, we decided to carry out more extensive and better controlled experiments in which the animals after receiving repeated inoculations of inactive elementary bodies or virus-free extracts of dermal vaccine virus were tested for resistance to infection with a weak virus as well as with a very potent one.

10 rabbits were used. Of these, 3 received repeated inoculations of a suspension of elementary bodies inactivated by addition of 0.3 per cent formaldehyde, 3 received virus-free extract of dermal virus, 2 control rabbits were injected with meat infusion broth and 2 with typhoid vaccine. The materials were prepared, and the inoculations were made over a period of 6 weeks as described above under Methods and Materials. 2 weeks after the last set of injections the rabbits were tested for resistance to 2 kinds of active virus administered dermally, intradermally, and intratesticularly. A second inoculation of the potent virus was given 2 weeks later. Each time the treated rabbits were inoculated with active virus, normal control rabbits also received similar inoculations. Serum was collected from each rabbit before treatment and at different times during the experiment for the study of humoral antibodies.

When one examines the results of Experiment 3, summarized in Table III, it is immediately obvious that none of the rabbits possessed humoral antibodies against vaccine virus at the beginning of the experiment and that the repeated injections of meat infusion broth or of typhoid vaccine did not produce antiviral antibodies or resistance to vaccinia in the animals receiving them. The rabbits that were treated with formolized elementary bodies or with virus-free extracts of dermal virus developed fair agglutinin and precipitin titers ranging

TABLE III

Results of Inoculation of Rabbits with Elementary Bodies, Inactivated by Means of 0.3 Per Cent Formaldehyde, and with Virus-Free Filtrates of Dermal Vaccine Virus

Rabbit No.	Serum*			Inocula	Serum*			Virus inoculation†					Serum*			Virus rein-oculation†		Serum*		
	Agglutination	Precipitation with testicle filtrate	Neutralization‡		Agglutination	Precipitation with testicle filtrate	Neutralization‡	Strain	Titer—intradermal	Titer—scarification	Testicular reaction	Maximum temperature	Agglutination	Precipitation with testicle filtrate	Neutralization‡	Strain	Titer—intradermal	Agglutination	Precipitation with testicle filtrate	Neutralization‡
												°F.								
0-39	—	—	—(—)	Elementary bodies	512	16	10(10)	C	0	0	0	103.4	512	16	10(10)	BH	10^5	256	16	(10^3)
0-40	—	—	—(—)	" "	256	8	$10^2(1)$	C	10^1	0	0	103.6	128	2	$10^2(1)$	"	10^5	128	8	(10^2)
0-41	—	—	—(—)	" "	256	16	10	BH	10^6	10^4	±	105.2	512	32	10^5	"	10^3	512	16	10^4
0-47	—	—	—(—)	Virus-free filtrate	256	16	10(10)	C	10^3	0	±	103.0	128	16	10(10)	"	10^5	128	16	(10^3)
0-50	—	—	—(—)	" "	128	8	1(0)	C	10^5	0	+	104.0	128	16	$10^4(10^4)$	"	10^2	128	8	(10^4)
0-81	—	—	—(—)	" "	64	16	1(1)	BH	10^6	10^4	+	104.4		Died						
0-58	—	—	—	Broth	—	—	—	C	10^5	10^2	+	105.4	256	16	$10^5(10^2)$	"	10^1	256	8	(10^2)
0-59	—	—	—	"	—	—	—	C	10^5	10^2	+	104.2	256	8	$10^4(10^4)$	"	10^1	256	16	(10^4)
0-63	—	—	—	Typhoid vaccine	—	—	—	C	10^4	10^2	+	105.2	256	8	$10^3(10^3)$	"	10^1	256	16	(10^3)
0-64	—	—	—	" "	—	—	—	C	10^5	10^2	+	104.4	256	8	10	"	10^2	256	8	10^4
1-99				None				C	10^5	10^2	+	105.1	64	4	10^5	"	10	64	4	10^5
2-00				"				C	10^5	10^2	+	105.1	64	16	10^6	"	10	64	16	10^6
2-01				"				C	10^5	10^2	+	105.8	256	16	10^4	"	10^2	128	16	
2-02				"				C	10^5	10^2	+	105.8	128	8	10^5	"	10	256	8	
2-69				"												"	10^6	512	8	10^5
2-25				"				BH	10^6	10^5	+	107.0		Died						

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ All samples of serum from a single animal were tested on the same rabbit. Results of duplicate tests, made on another rabbit, are bracketed in order to facilitate comparison of the results.

from 1:64 to 1:512 and from 1:8 to 1:16, respectively. The neutralizing titers of the same sera, however, were very low, inactivating only 1 to 10 infectious doses of active virus. It is true that one of these sera (0-40) inactivated 100 doses of virus in one test, but when investigated again it neutralized only 1 dose.

One of the 3 rabbits that had been treated with inactive elementary bodies showed no evidence of infection when inoculated dermally, intradermally, and intratesticularly with culture virus, an agent of weak pathogenicity for rabbits. Another animal of this group similarly inoculated showed a vaccinal lesion only at the point where the lowest dilution of the culture virus was introduced intradermally. The third animal of the group received dermal, intradermal, and intratesticular inoculations of BH virus, an agent of strong pathogenicity for the rabbit, and showed only a slight amount of resistance to infection. In fact, it was necessary to compare the lesions in the treated rabbit with those in a control in order to appreciate that the inoculations of inactive elementary bodies had modified the course of the infection.

Of the 3 rabbits treated with virus-free extracts, 2 were tested with active culture virus and 1 with the BH virus and evidenced much less resistance than did those that had been treated with inactive elementary bodies. No lesions occurred at the sites of dermal inoculation of culture virus. Lesions did occur, however, as the result of intratesticular and intradermal injections of the active agent, and one of the animals had fever. Although 10^3 and 10^5 dilutions of the virus produced lesions when injected intradermally such lesions were definitely different from those that occurred in controls. The rabbit that was tested with the BH virus showed no evidence of having been benefited by the treatments with virus-free extracts; in fact, it died of the vaccinal infection.

Samples of serum collected from treated and control rabbits 2 weeks after the first inoculations of active virus gave interesting results when tested for humoral antibodies. All of the control animals showed good titers of agglutinins, precipitins, and neutralizing antibodies. Rabbit 0-41 that received active BH virus after treatment with inactive elementary bodies and developed a definite infection, showed a rise in the amount of all humoral antibodies, and animal

0-50 that received active culture virus after treatments with virus-free extracts and developed an infection, also showed a marked rise in its neutralizing antibodies. The other treated rabbits (0-39, 0-40, 0-47) after inoculations of the active culture virus showed either no change or a decrease in the amounts of agglutinins, precipitins, and neutralizing antibodies.

In order to determine whether the findings just described were due to defects in the methods of testing the humoral antibodies or represented a failure of the weak culture virus to enhance a slight amount of immunity already present, the animals were reinoculated with the potent BH virus. The rabbits that had responded to the first inoculations of active virus with evident infections manifested on this occasion a fairly high degree of resistance with lesions only at the sites of inoculation of 1:100 and 1:1000 dilutions of the virus and no increase in their titers of neutralizing antibodies. On the other hand, the treated rabbits, previously refractory to active weak culture virus, developed lesions at the sites of inoculation of 1:100,000 dilutions of the BH virus; necrotic areas appeared in the center of the lesions caused by undiluted virus and that diluted ten times; and there was a sharp rise in the neutralizing titers of the sera. In comparing the neutralization titers of samples of serum from rabbit 0-40 only the bracketed figures should be considered at this time.

When one examines the results (Table III) obtained by inoculating normal rabbits with culture virus and compares them with the ones just described it seems that a weak immunity induced by inactive virus or virus-free material can at times interfere or prevent an enhancement of this slight amount of resistance by means of a weak active agent.

Experiment 4

In order to determine whether an increase in the length of time that the elementary bodies remained in contact with the 0.3 per cent formaldehyde or whether a moderate increase in the concentration of formaldehyde used for inactivation of the virus would yield results different from those obtained in Experiment 3, another experiment was carried out in the following manner.

TABLE IV

Results of Inoculation of Rabbits with Elementary Bodies Which Had Been Suspended in 0.3 Per Cent Formaldehyde for 3½ Months and 1.0 Per Cent Formaldehyde for an Additional 10 Days

Rabbit No.	Serum* Agglutination	Serum* Precipitation with dermal filtrate	Serum* Precipitation with testicle filtrate	Serum* Neutralization	Inocula	Serum* Agglutination	Serum* Precipitation with dermal filtrate	Serum* Precipitation with testicle filtrate	Serum* Neutralization	Virus inoculation† Strain	Virus inoculation† Titer—intradermal	Virus inoculation† Titer—scarification	Virus inoculation† Testicular reaction	Virus inoculation† Maximum temperature	Serum* Agglutination	Serum* Precipitation with dermal filtrate	Serum* Precipitation with testicle filtrate	Serum* Neutralization	Virus reinoculation† Strain	Virus reinoculation† Titer—intradermal	Virus reinoculation† Testicular reaction	Virus reinoculation† Maximum temperature	Serum* Agglutination	Serum* Precipitation with dermal filtrate	Serum* Precipitation with testicle filtrate	Serum* Neutralization
														°*F.*								°*F.*				
2-50	—	—	—	—	Elementary bodies 0.3% formaldehyde	256	4	4	10^1	C	10^1	—	—	103.1	128	—	—	10^2	BH	10^4(E)	—	103.8	128	4	8	10^6
2-51	—	—	—	—	" "	256	4	8	10^5	BH	10^3	10^1	±	103 1	256	8	16	10^5	BH	10^4(E)	—	102 9	128	8	16	$\cancel{10^5}$
2-52	—	—	—	—	Elementary bodies 1.0% formaldehyde	128	1	4	10^1	C	10^2	10^1	—	103.0	64	—	2	10^4	BH	10^4(E)	—	102 9	128	4	32	10^5
2-53	—	—	—	—	" "	256	4	16	1	BH	10^5	10^2	—	103 2	256	4	16	10^4	BH	10^3(E)	—	‡	256	16	32	10^5
2-56	—	—	—	—	Broth	—	—	—	—	C	10^5	10^2	+	103 6	128	4	8	10^6	BH	10^1	±	103.2	128	8	16	10^6
2-54	—	—	—	—	"	128	4	8	10^4	BH	10^1	10^1	—	102 4	64	2	8	10^4	BH	10^3	±	103.2	256	32	16	10^5
2-76	—	—	—	—	None	—	—	—	—	C	10^5	10^2	+	105 2	256	2	8	10^5	BH	10^3	+	103.4	128	8	16	10^5
2-57	—	—	—	—	"	—	—	—	—	BH	10^5	10^4	+	106.6	256	8	32	10^6	BH	10^1	—	103.0	512	32	32	10^6
4-59					"					C	10^2	10^2	+	105 0	128	2	16	10^5	BH	10^4(E)	—	102 5				10^5
4-58					"					BH	10^5	10^3	+	105 4	Died											
5-10					"										—	—	—		BH	10^6	+	104 2				

(E), marked edema at site of injection, subsiding in 48 to 72 hours.

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ Animal developed snuffles with fever; temperature readings, therefore, are not significant.

A portion of the elementary body suspension prepared for Experiment 3 was employed; the virus had been in contact with 0.3 per cent formaldehyde for a period of over 3 months. The material was divided into 2 portions. To one of them sufficient formaldehyde was added to increase the final concentration from 0.3 to 1.0 per cent. After a period of 10 days the suspensions were used for the repeated intraperitoneal inoculation of rabbits according to the schedule used in the previous experiment. 2 rabbits were used for each of the preparations of elementary bodies, 2 rabbits received meat infusion broth, and 2 rabbits received no injections. Samples of serum were collected and studied, and the animals were tested twice for immunity to active virus as in Experiment 3.

An examination of the results of the above experiment, summarized in Table IV, reveals that they are similar to those recorded for Experiment 3 and indicate that contact of elementary bodies of vaccinia with 0.3 per cent formaldehyde for $3\frac{1}{2}$ months or contact with 0.3 per cent formaldehyde for over 3 months followed by contact with 1.0 per cent of it for 10 days does not completely destroy their antigenicity. Inasmuch as the results are similar to those of Experiment 3, a detailed discussion of them will be omitted.

Two points of special interest are found in Table IV. 5 of the rabbits, Nos. 2-50, 2-51, 2-52, 2-53, and 4-59, rapidly developed a marked edema, which subsided within 48 to 72 hours, at the sites of the second set of inoculations of active virus. After the disappearance of the edema, the vaccinal lesions seemed to proceed in the usual manner. Inasmuch as antiviral antibodies appeared in its serum during the time that only injections of broth were being administered, it is obvious that rabbit 2-54 became accidentally infected with vaccinia in spite of precautions taken to prevent such an occurrence. Moreover, the high neutralizing titer (10^5) of the serum of rabbit 2-51 that received elementary bodies inactivated by 0.3 per cent formaldehyde and the relatively good resistance to potent BH virus subsequently evidenced by the animal, lead one to suspect that an accidental infection with active virus also occurred in this rabbit.

Experiment 5

The experiments described above demonstrated that moderate amounts of formaldehyde decrease but do not completely destroy the antigenicity of the elementary bodies of vaccinia. It seemed desirable therefore, to ascertain whether more drastic treatment with formaldehyde or heat is capable of effecting such a destruction.

A suspension of highly purified elementary bodies was prepared and divided into 2 portions. One portion was boiled for 2 hours. The elementary bodies in

the other portion were sedimented by centrifugation and resuspended in a small amount of 10 per cent formaldehyde. After 10 days the formolized suspension was diluted with 10 times its volume of buffer solution. 2 rabbits received repeated intraperitoneal inoculations of the formolized elementary bodies, 2 were inoculated with the boiled elementary bodies, and 2 that were given virus-free extracts prepared from emulsions of tissues containing herpetic virus served as controls. Inasmuch as the suspension of elementary bodies was more concentrated

TABLE V

Results of Inoculation of Rabbits with Elementary Bodies Inactivated by Means of 10 Per Cent Formaldehyde or by Boiling

Rabbit No.	Serum*			Inocula	Serum*			Virus inoculation†					Serum*		
	Agglutination	Precipitation with testicle filtrate	Neutralization		Agglutination	Precipitation with testicle filtrate	Neutralization	Strain	Titer—intradermal	Titer—scarification	Testicular reaction	Maximum temperature	Agglutination	Precipitation with testicle filtrate	Neutralization
												°F.			
3-44	–	–	–	Formolized elementary bodies	32	–	–	BH	10^4	10^2	–	104.5	256	16	10^6
3-45	–	–	–	" "	2	–	–	C	10^3	10^2	±	102.8	128	4	10^3
3-46	–	–	–	Heated elementary bodies	2	–	–	BH	10^4	10^4	+	106.0	128	16	10^6
3-47	–	–	–	" "	8	–	–	C	10^3	10^1	–	103.8	64	4	10^3
3-54	–	–	–	Herpes virus extract plus swine serum	–	–	–	BH	10^5	10^5	+	105.4	256	8	10^6
3-56	–	–	–	" "	–	–	–	C	10^1	10^1	–	‡	2	2	10^6
5-00				None				BH	10^4	10^4	+	105.8	512	16	10^4
5-02				"				C	10^3	10^1	+	102.8	64	–	10^3
5-01				"				BH	10^5	10^5	+	106 4	256	16	10^4
5-03				"				C	10^3	10^2	–	101.8	32	1	10^3

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ Animal developed snuffles; temperature determinations, therefore, are not significant.

than those previously used, each rabbit received only 12 cc. of material given in 6 doses over a period of 3 weeks. Serum was collected from all the rabbits at the beginning of the experiment and at different times during it. 2 weeks after completion of the series of injections the treated rabbits and normal controls were tested for resistance to active BH and culture viruses.

The results of Experiment 5, summarized in Table V, show that repeated inoculations of elementary bodies that had been boiled or

treated with 10 per cent formaldehyde did not induce in rabbits the production of precipitins, neutralizing antibodies, or resistance to active vaccine virus. However, a few agglutinins, titers ranging from 1:2 to 1:32, did appear in the sera of the inoculated rabbits.

Experiment 6

It has been stated (34) that virus-free extracts of testicular vaccine virus although containing substances precipitable by antivaccinal serum, do not induce the production in rabbits of antiviral antibodies. In Experiments 1 and 3, however, we found that animals repeatedly inoculated with virus-free extracts of dermal vaccine virus developed humoral antibodies and in certain instances a slight amount of resistance to infection with vaccinia. Inasmuch as some workers have suggested (34) that the precipitinogen in these extracts is a haptene, it occurred to us that perhaps an incomplete antigen or haptene in the dermal extracts was completed by the foreign protein liberated by the bacteria inevitably present in dermal virus. We tested this idea by using as inocula virus-free extracts containing the precipitinogen or precipitinogens prepared from testicular vaccine virus free from bacterial contaminants, and such extracts to which sterile swine serum had been added. Virus-free extracts of testicular herpes virus plus swine serum were used as control inocula.

An emulsion of testicular vaccine virus was prepared by grinding dried infected testicular tissue with Locke's solution; 100 cc. of fluid were used for each gram of dried material. After centrifugation the emulsion was passed through a Seitz filter and then a collodion membrane. The filtrate was shown to be virus-free. The material was divided into 2 portions. One portion was used for inoculation without further treatment. To the other was added sufficient sterile swine serum to make its concentration 10 per cent. As a control a similar preparation of herpes testicular virus was made to which pig serum was added. 3 rabbits received the vaccine virus extracts alone, 2 the vaccine virus extracts plus swine serum, and 3 the herpes virus extracts plus swine serum. Each rabbit received 12 cc. of material administered intraperitoneally in 6 doses over a period of 3 weeks. Samples of serum were collected at different times for the study of humoral antibodies. 2 weeks after the last inoculations, the animals were tested for resistance to infection with vaccinia, both culture and BH strains of virus being used.

The results of Experiment 6, summarized in Table VI, show that the animals receiving the extract of testicular herpes virus plus swine

serum developed no antivaccinal antibodies, while those that were given extracts of testicular vaccine virus or extracts of testicular vaccine virus plus swine serum only responded with the production of slight amounts—in some instances none—of agglutinins, precipitins, and protective or neutralizing antibodies. Furthermore, it is obvious

TABLE VI

Results of Inoculation of Rabbits with Virus-Free Filtrate of Testicular Vaccine Virus Extract and Virus-Free Filtrate of Testicular Virus Extract Plus Swine Serum

Rabbit No.	Serum*				Inocula	Serum*				Virus inoculation†					Serum*			
	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization		Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization	Strain	Titer—intradermal	Titer—scarification	Testicular reaction	Maximum temperature	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization
														°F.				
3-48	–	–	–	–	Testicle virus extract	8	–	–	–	BH	10^5	10^3	+	104 0	128	16	32	10^5
3-49	–	–	–	–	" "	8	–	2	–	BH	10^4	10^4	+	107.0		Died		
3-50	–	–	–	–	" "	256	2	8	–	C	10^3	10^1	+	102.3	128	16	16	10^1
3-51	–	–	–	–	Testicle virus extract plus swine serum	8	–	–	–	BH	10^4	10^3	+	105.8		Died		
3-52	–	–	–	–	" "	–	–	–	1	C	10^3	10^1	+	107.2	–	4	2	10^5
3-54	–	–	–	–	Herpes virus extract plus swine serum	–	–	–	–	BH	10^6	10^5	+	105.4	256	8	8	10^6
3-55	–	–	–	–	" "	–	–	–	–	BH	10^4	10^4	+	105.0		Died		
3-56	–	–	–	–	" "	–	–	–	–	C	10^1	10^1	–	‡	2	–	2	10^6
5-00					None					BH	10^4	10^4	+	105.8	512	8	16	10^4
5-01					"					BH	10^5	10^5	+	106.4	256	4	16	10^4
5-02					"					C	10^3	10^1	+	102.8	64	2	–	10^2
5-03					"					C	10^3	10^2	–	101 8	32	2	1	10^3

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ Animal developed snuffles; temperature determinations are, therefore, not significant.

that the addition of swine serum to the extract of testicular vaccine virus did not make the latter material a better antigen for the stimulation of antivaccinal antibodies. None of the rabbits evidenced, as a result of the repeated inoculations, an appreciable degree of resistance to infection with vaccinia; in fact, 3 of them died. Accurate

readings of the reactions were difficult because of the marked spreading of the infection around the sites of inoculation which doubtless occurred in consequence of the rabbits having received large amounts of material containing the "spreading factor" of Duran-Reynals (35).

In Experiments 1 and 3 the rabbits that received inoculations of virus-free extracts of dermal vaccine virus produced more humoral antibodies and evidenced more resistance, even though it was not a great amount, than did the animals in Experiment 6 that were given injections of virus-free extracts of testicular vaccine virus. This discrepancy in the results of the two sets of experiments may be accounted for in two ways. Firstly, in Experiments 1 and 3 each rabbit received 15 and 18 cc. of dermal extracts, respectively, administered in 12 doses over a period of 6 weeks, while in Experiment 6 each rabbit received only 12 cc. of testicular extract administered in 6 doses over a period of 3 weeks. Secondly, according to our findings, the concentration of the precipitable vaccinal substances or antigens in the dermal extracts is greater than in the testicular extracts.

Experiment 7

Having determined that humoral antibodies and a certain amount of resistance to vaccinia can be produced in rabbits by the repeated injection of inactive formolized elementary bodies and virus-free preparations of the precipitable vaccinal antigens, we decided to investigate their persistence for a comparison with the phenomena that follow a frank infection with vaccine virus. To this end the following experiment was carried out.

The rabbits were treated with inactive formolized elementary bodies of vaccinia, virus-free filtrates of dermal vaccine virus, typhoid vaccine, and meat infusion broth in a manner identical with that employed in Experiment 3 with the exception that they were held for 114 days, instead of 14, after the series of inoculations before being tested for resistance to infection with active vaccine virus. 1 rabbit received inactive elementary bodies, 1 virus-free filtrate, 1 typhoid vaccine, and 1 broth. For comparison 2 rabbits were inoculated with active BH virus and held in another room. At the beginning, during, and at the end of the experiment samples of serum were collected from each rabbit for the estimation of humoral antibodies.

TABLE VII

Duration of Immunity in Rabbits Following Repeated Inoculations of Virus-Free Extracts of Dermal Vaccine Virus and Elementary Bodies Inactivated by Means of 0.3 Per Cent Formaldehyde

Rabbit No.	Serum*			Inocula	Serum* after										Virus inoculation†						Serum*		
					14 days			78 days		102 days		114 days											
	Agglutination	Precipitation with testicle filtrate	Neutralization		Agglutination	Precipitation with testicle filtrate	Neutralization	Agglutination	Precipitation with testicle filtrate	Agglutination	Precipitation with testicle filtrate	Agglutination	Precipitation with testicle filtrate	Neutralization	Strain	Titer—intradermal	Testicular reaction	Maximum temperature	Strain	Titer—intradermal	Agglutination	Precipitation with testicle filtrate	Neutralization
																		°F.					
0-46	—	—	—	Formolized elementary bodies	256	8	10^4	32	2	8	2	16	—	10^2	BH	10^5	+	105 0	C	10^4	128	32	10^5
0-82	—	—	—,—	Virus-free filtrate	64	16	10,10	4	1	2	—	2	—	0,0	BH	10^5	+		C	10^2	Died		
0-62	—	—	—	Broth	—	—	—	—	—	—	—	—	—	—	BH	10^5	+	106 2	C	10^4	128	4	10^5
0-65	—	—	—	Typhoid vaccine	—	—	—	—	—	32	8	16	1	10^4	BH	10^2	—	103 3	C	—	16	2	10^5
2-69				Active BH virus	512	8	10^5					128	4	10^4	BH	10^1	—	103 0			128	16	10^4
2-70				" " "	512	16	10^4					256	2	10^4	BH	10^1	±	103.8			64	2	10^4
5-00				None											BH	10^4	+	105 8			512	16	10^4
5-01				"											BH	10^5	+	106 4			256	16	10^4
5-02				"															C	10^3	64	—	10^2
5-03				"															C	10^3	32	1	10^3

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

From the results of Experiment 7, summarized in Table VII, it is at once apparent that none of the animals possessed humoral antibodies at the beginning of the experiment and that one of the control rabbits, No. 0-65, that received the typhoid vaccine was accidentally infected with active vaccine virus in spite of our efforts to prevent such an occurrence. As a result of repeated injections of inactive elementary bodies and virus-free filtrates, respectively, in rabbits 0-46 and 0-82, considerable amounts of humoral antibodies, more in the former animal than in the latter, were developed, all of which were gradually decreasing in amount when last investigated 114 days after completion of the series of injections. Both of these rabbits when inoculated with active BH and culture viruses seemed fully susceptible to infection. In the control rabbits 2-69 and 2-70 that received the BH virus at the beginning of the experiment, the agglutinins and precipitins, but not the neutralizing antibodies, also gradually decreased in amount during the detention period of 114 days. However, when these rabbits were reinoculated with active BH virus, an almost complete resistance to infection was exhibited.

It is obvious that too few rabbits were used in Experiment 7 to warrant definite conclusions. At that time more animals could not be properly housed in our isolation room. Nevertheless, when one examines the results of Experiments 3 and 7, it appears that the slight amount of resistance to vaccinal infection produced in rabbits by repeated injections of inactive elementary bodies or virus-free filtrates of dermal virus tends to disappear rapidly, while that caused by active virus is more enduring.

DISCUSSION

In the study of infectious diseases the importance of an accurate knowledge of the response of animals to the inciting agents in an inactive as well as active state has long been recognized. Such information leads to a better insight into the mechanism of immunity in which multiplicity, lability, and stability of antigens, phenomena of infection, and intimacy, duration, and possibly persistence of infection play important rôles. With this increase of knowledge regarding immunity one may reasonably expect the appearance of opportunities to prevent certain diseases by means of vaccines made of inactive

or attenuated agents or to improve methods of vaccination already in use.

As pointed out earlier in the paper many investigations along the lines referred to have been made with vaccine virus, but, as indicated, practically all of the work is subject to criticism for one or more reasons. Recent advances in the knowledge of vaccine virus have made it possible for us to eliminate certain of the sources of error by the use of preparations of highly purified elementary bodies and virus-free extracts of virus-infected tissues containing specific vaccinal antigens. Great care was taken to be as certain as now is possible that no active virus remained in the preparations spoken of as inactive. Furthermore, in view of the ease with which rabbits are accidentally infected with active vaccine virus, rigid isolation precautions were observed. In spite of such precautions, 2 control rabbits and probably 1 test animal were accidentally infected with vaccinia. It is obvious, however, from the results shown in the seven tables that these accidental infections do not invalidate the conclusions we have drawn. Nevertheless, they emphasize the fact that any work of this nature conducted with inactive vaccine virus in the absence of rigid isolation is valueless.

It is apparent from the data presented that the repeated injections in rabbits of large quantities of elementary bodies of vaccinia inactivated by small amounts of formaldehyde (0.3 per cent) led to the appearance in the serum of specific agglutinins, precipitins, and neutralizing antibodies as well as to the development in the animals of a certain degree of resistance to infection with vaccinia. The immunity thus induced was slight and apparently not enduring, and was best demonstrated by the use of a strain of virus of low pathogenicity for the rabbit. A similar but less marked response followed the repeated injections of virus-free extracts of dermal vaccine virus. Furthermore, the inactive elementary bodies and virus-free extracts were less efficient antigens when similar amounts were given in single large doses instead of repeated small ones. Finally, drastic treatment (10 per cent formaldehyde or boiling for 2 hours) almost completely altered or destroyed the antigenicity of elementary bodies.

In a certain number of the rabbits that had received repeated injections of inactive elementary bodies or virus-free extracts (Experi-

ment 3) little or no apparent infection resulted from dermal, intradermal, and intratesticular inoculations of active culture virus. Nor was there an appreciable increase in the amount of neutralizing antibodies in the serum of the animals after they had received this active virus. 2 weeks after this test for resistance to infection with culture virus, another test was made with the highly pathogenic BH virus, at which time the rabbits were found still to be moderately susceptible to vaccinia. These results are different from those obtained with normal rabbits in which a good resistance to the BH strain of virus is developed as a result of inoculations ot active culture virus. The inhibition phenomenon just described did not occur regularly and the mechanism of its production is not known.

Recently, Craigie (36) has reported that there are at least two antigens in vaccine virus, one labile (L), the other stable (S). According to him, both antigens incite the production of agglutinins and precipitins. Furthermore, he suggests that the L antigen may function in the production of resistance to vaccinal infection. In view of the description of his methods, it is not unlikely that we destroyed all or most of the L antigen in our elementary bodies by the process of inactivation. In any event, our preparations seemed to have caused no infection, and with them we obtained considerable amounts of humoral antibodies, including neutralizing ones, and some resistance to infection. Such results are not surprising because as yet there is no reason (37) to suppose that vaccine virus does not contain protein, and the injection of enough foreign protein into a rabbit should induce the animal to respond in some manner. It remains to be seen, however, whether with methods of inactivation less drastic than those used by us a truly noninfectious virus can be obtained the repeated injections of which will cause a greater degree of resistance to infection.

We found no evidence in our data to suppose that agglutinins and precipitins for vaccine virus are different antibodies. Our results have been interpreted as showing that agglutination and precipitation are due to the same antibody, or, if not, to different antibodies the production of which was not independently stimulated. On the other hand we observed that the agglutinating and precipitating activity of the sera did not necessarily parallel their virus neutralizing

properties; a high titer of agglutinins and precipitins was at times associated in the same serum with a slight amount of neutralizing antibodies and *vice versa*. Furthermore, the presence of considerable amounts of neutralizing antibodies could not be taken as an indication that the animals were protected against infection.

If our elementary bodies were really inactive—there is no reason to suppose that they were not—, then the neutralizing antibodies resulting from the repeated injections of them into rabbits could not have been induced by an antigen produced by the hosts as a result of a vaccinal infection (38). One might suppose that such an antigen, having arisen in the animals from which the elementary bodies were obtained, had not been completely removed from the elementary bodies by repeated washing and was still operative. All one can say to such a suggestion is that the virus-free filtrates of the material from which the elementary bodies were obtained and which should have contained large quantities of the hypothetical antigen engendered by the host as a result of infection were considerably less effective in inciting the production of neutralizing antibodies than were the washed elementary bodies.

Although the results of our experiments show that a certain amount of resistance to vaccinia, probably not enduring, can be secured in rabbits by the use of inactive elementary bodies, there is still no reason to suppose that the use of such materials is suitable for the protection of human beings against smallpox. Each rabbit that developed resistance to vaccinia received all of the elementary bodies, suspended in 12 to 18 cc. of fluid, that were obtained from another rabbit infected over a large area of skin (32). Consequently, if the same amount of inactive elementary bodies per body weight were required to induce the same amount of resistance in man as in the rabbit, several hundred cubic centimeters of a very expensive vaccine would be required. And then we would have no assurance that the vaccinated individuals would be protected against smallpox.

SUMMARY AND CONCLUSIONS

Humoral antibodies and a certain degree of resistance to infection with vaccinia, probably not enduring, are produced in rabbits by the repeated injections of inactive formolized (0.3 per cent) elementary

bodies of vaccinia and virus-free filtrates of dermal vaccine virus. Single injections of large amounts of elementary bodies are not as effective as similar amounts administered in small repeated doses. Drastic treatment (10 per cent formaldehyde or boiling for 2 hours) almost completely alters or destroys the antigenicity of elementary bodies.

It appears that the production of precipitins and agglutinins does not parallel that of neutralizing antibodies and that the mere presence of such antibodies in the serum of a rabbit as the result of injections of inactive elementary bodies does not necessarily indicate that the animal possesses a great degree of resistance to infection with a potent vaccine virus.

The fact that some neutralizing antibodies appeared in the sera of rabbits that had received injections of inactive elementary bodies can be interpreted as indicating that at least not all neutralizing antibodies for vaccine virus are the result of a reaction to an antigen produced by the host in consequence of a vaccinal infection.

No evidence was obtained to show that elementary bodies inactivated by our methods (0.3 per cent formaldehyde) would serve as a suitable vaccine for the protection of human beings against smallpox.

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PLASMA LIPIDS OF NORMAL MEN AT DIFFERENT AGES*

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The present paper has two objects: first, to ascertain the effect of age, beginning with the third decade, on the plasma lipid content of normal men; second, to ascertain the normal ranges, in adult men, of the different lipid fractions determined by the gasometric methods of Kirk, Page, and Van Slyke (1934).

Subjects and Blood Sampling—The subjects were men who by ordinary clinical examination were healthy. They were on unrestricted diets. Blood was drawn before breakfast, and was at once heparinized and centrifuged. The plasma and red cells were carefully separated to avoid the inclusion of platelets and white cells. The lipids of the plasma were immediately extracted with alcohol-ether, and the different lipid fractions were determined by duplicate analyses, as described by Kirk, Page, and Van Slyke (1934).

Calculation of Data—To render the results into usual terms, and to facilitate comparison with the results of previous authors, we have, from the directly determined values of lipid carbon, phosphorus, etc., calculated the different plasma lipids in conventional units by the following formulæ:

(1) $$\textit{Phosphatides} = 23.5 \times (\text{total lipid P})$$

* The analyses have been carried out by Page and Kirk, with the technical assistance of Mr. Howard Read. The subjects over 40 years of age were selected and subjected to clinical examination by Lewis, in connection with a study of age changes being carried on by Dr. Alfred E. Cohn's department in the Hospital of the Rockefeller Institute. The statistical analysis of the age groups presented in Tables IV and V was carried out by Thompson.

See Table X of Kirk, Page, and Van Slyke (1934) and its discussion for the factor 23.5.

(2) *Esterified cholesterol* = (total cholesterol) − (free cholesterol)
(3) *Cholesterol esters* = 1.69 (esterified cholesterol)

The factor 1.69 is approximately exact for cholesterol stearate and oleate. (In this paper "esterified cholesterol" is used to indicate the amount of cholesterol, $C_{27}H_{46}O$, which is in the ester form, while "cholesterol esters" is used to indicate the amount of the esters, of formula $C_{27}H_{45} \cdot O_2C_{18}H_{35}$ in the case of the stearate.)

(4) *Neutral fat C* = (total lipid C) − [15.5 (lipid P) + 0.839 (free cholesterol) + 1.40 (esterified cholesterol)]

The *neutral fat C* is calculated in Equation 4 by subtracting from the total lipid C the phosphatide C, calculated as 15.5 P, the free cholesterol C, and the cholesterol ester C, represented by the successive terms of the equation. The C:P ratio, 15.5, used for the phosphatides is the ratio found by Kirk, Page, and Van Slyke ((1934) see their Table X) in plasma phosphatides. It approximates the ratio, 15.8, which is theoretical for cephalin containing two fatty acid radicals with 18 carbon atoms each. The factor 0.839 represents the C content of free cholesterol. The calculation of cholesterol ester C as 1.40 × (esterified cholesterol) is theoretical for esters of cholesterol with 18-carbon fatty acids.

We have followed the convention of previous authors (*e.g.*, Boyd (1933)) in using the term "neutral fat" to indicate the plasma lipids found in excess of the phosphatides, cholesterol, and cholesterol esters. The material so defined may contain, besides simple triglycerides, unidentified petroleum ether-soluble material of other nature. While, therefore, for convenience and conformity with usage we employ the term "neutral fat" for this fraction, we wish to emphasize that by this usage we do not imply any assumption concerning the proportion of simple triglycerides in the fraction. A more adequate term for the fraction would be "neutral fat and unidentified petroleum ether-soluble extractives."

(5) *Neutral fat* = 1.32 (neutral fat C)

The factor 1.32 is theoretical for glyceryl tristearate.

Total lipids have been calculated in two ways: first, by sum-

mation of the separate lipid fractions, and, second, by multiplying the directly determined total lipid carbon by the factor 1.3, found by Kirk, Page, and Van Slyke (1934) to give an approximately accurate value for the total mixture. Equations 6 and 7 are used for the two types of calculation.

(6) *Total lipids* = phosphatides + free cholesterol + cholesterol esters + neutral fat

The phosphatides, cholesterol esters, and neutral fat are calculated by Equations 1, 3, 4, and 5.

(7) *Total lipids* = 1.3 (total lipid C)

Theoretically, as pointed out by Kirk, Page, and Van Slyke (1934), calculation of total lipids by summation (Equation 6) should be more exact than direct calculation from total lipid C (Equation 7), because the factor 1.3 is exact only for a mixture with 77 per cent of carbon. It is almost exact for the usual triglycerides, but would be too high if cholesterol, with 83.9 per cent C predominated, and would be too low if phosphatides, with 65 to 66 per cent C, formed most of the mixture. Actually, however, the results show that the total lipid values calculated by the two methods seldom differ by as much as 2 per cent, and show an average difference of less than 1 per cent. Both calculations involve approximate constants (Equation 6 is based partly on Equation 5), but neither appears subject to important errors. Because the results by Equations 6 and 7 are nearly identical, we give in the tables only the total lipid values calculated by Equation 6.

The results with normal subjects are given in Tables I, II, and III.

STATISTICAL ANALYSIS

Symbols

$s = \sqrt{\Sigma(x_i - \bar{x})^2/(N - 1)}$ *Fisher's Standard Deviation*—This standard deviation formula was apparently introduced by Gauss, but has been brought into general use by Fisher (1932). It is preferred to the "sample standard deviation" $\sqrt{\Sigma(x_i - \bar{x})^2/N}$ because, with finite N values, the Fisher formula is believed to yield values more likely to approximate the true standard deviation that would be yielded by either formula with an infinite

TABLE I

Plasma Lipids in Normal Men; Ages 21 to 49 Years

Plasma No.	Subject	Age	Lipids, mg. per 100 cc. plasma										
			Directly determined data							Calculated lipids			
							Cholesterol						
			Total lipid C	Total lipid P	Total lipid N	Lipid amino N	Total	Free	Esterified Equation 2	Cholesterol esters Equation 3	Phosphatides Equation 1	Neutral fats, etc. Equations 4, 5	Total lipids Equation 6
		Age class 20–39											
		yrs.											
1-a	Mu	21	547	7.7	19.2	5.3	112	81	31	53	182	419	730
1-b	"	21	448	4.3	6.2	3.3	214	73	141	238	102	162	575
2	Di	27	322	3.4	4.3	1.7	139	69	70	118	80	149	416
3-a	Ki	28	568	10.2	21.4	4.9	208	82	126	213	240	219	754
3-b	"	28	534	5.3	9.3	3.3	235	80	155	262	125	248	715
4-a	Be	29	355		6.1	0.9	109	64	45	84			462*
4-b	"	29	510	5.1	8.3	1.5	172	78	94	159	121	313	671
5	Mi	29	601	1.8	6.8	2.2	341	110	231	390	41	211	752
6	Ab	29	496	6.8	11.8	3.1	221	73	148	250	161	169	653
7	Le	31	515	8.5	9.2	3.8	262	86	176	297	201	91	675
8	Al	33	454	5.6	8.6	3.5		77			131		590*
9	Pa	34	788		6.8	4.4	237	98	139	235			1024*
10	Ho	35	728	10.2	8.0	2.9	299	107	192	324	240	281	952
		Age class 40–44											
11	Gr	40	643	10.7	14.1	4.5	239	105	134	226	253	264	848
12	Do	41	460	7.4	12.7	3.3	176	58	118	199	175	172	604
13	Io	41	447	5.8	6.3	0.5	219	74	145	245	137	124	580
14	El	44	614	10.8	11.7	5.3	230	96	134	226	255	235	812
15	Mc	44	433	4.9	8.8	1.1	237	82	155	262	116	94	554
		Age class 45–49											
16	Cr	45	440	4.2	6.9	0.8	264	74	190	321	99	62	556
17	Ed	47	515	5.3	8.1	2.2	215	67	148	250	125	224	666
18	Co	47	354	2.7	12.2	1.1	218	66	152	257	64	58	445
19	Ja	48	522	9.0	14.2	3.2	250	66	184	311	212	92	681
20	Dy	49	528	9.7	12.6	4.7	318	96	222	375	229	(0)†	700

* Total lipids were calculated by Equation 7, because of lack of phosphatide or cholesterol figures for calculation by Equation 6.

† The figure in parentheses (Subject 20) is rejected in calculations of mean, standard deviation, and normal range.

Table II

Plasma Lipids in Normal Men; Ages 50 to 69 Years

Plasma No.	Subject	Age	Lipids, mg. per 100 cc. plasma: Directly determined data: Total lipid C	Total lipid P	Total lipid N	Lipid amino N	Cholesterol: Total	Free	Esterified Equation 2	Calculated lipids: Cholesterol esters Equation 3	Phosphatides Equation 1	Neutral fats, etc. Equations 4, 5	Total lipids Equation 6
						Age class 50–54							
		yrs.											
21	Ho	50	538	10.2	5.8	1.2	188	69	119	201	240	205	715
22	To	51	468	9.1	10.4	3.1	188	65	123	208	215	132	620
23	Wi	51	401	5.7	12.1	4 2	173	70	103	174	135	145	524
(24)	Da	51	1780	13.6	18.4	4.4	428	135	293	495	321	1390	2341
25	Ro	52	772	8.2	11.4	3.7	336	102	234	395	194	305	996
26	Wi	54	551	4.6	4.0	(0.2)	213	60	153	258	112	285	715
						Age class 55–59							
27	Ne	55	389	3.8	5.4	0.9	169	68	101	171	90	174	503
28	McM	55	405	4.4	8.2	0.8	208	62	146	246	105	107	520
29	Nu	56	481	2.7	(1.7)	0.9	211	68	143	242	63	240	613
30	Bi	57	555	8.0	10.8	2.2	190	70	120	203	189	269	731
31	MaC	58	828	11.4	16.7	4 9	286	89	197	332	269	396	1086
32	Po	59	686	5.5	13.1	3.2	234	74	160	270	130	416	890
33	McC	59	1007	9.3	8.6	2.4	293	84	209	353	220	662	1319
34	Ri	59	1095	6.4	7.9	3.2	376	110	266	449	151	703	1413
						Age class 60–64							
35	Ro	60	836	13.7	19.4	8.2	351	102	249	421	321	249	1093
36	Go	63	430	8.3	12.1	2.7	152	84	68	115	196	179	574
37	Ed	63	402	4.1	5.8	0.8	206	94	112	189	97	136	516
38	Ka	64	465	6.9	8.5	4.0	213	104	109	184	163	157	608
39	Sh	64	641	9.8	12.2	5.2	273	78	195	330	231	198	837
40	La	64	438	(0.7)	3.2	1.9	284	76	208	351	(17)	95	539
						Age class 65–69							
41	Pi	65	444	7.2	14.8	3.2	206	59	147	248	170	100	577
42	So	66	482	8.4	23.9	3.9	205	126	79	133	198	178	635
43	Wi	68	636	11.0	18.6	5.1	280	73	207	350	260	145	828
44	Sm	69	536	9.3	13.3	5.3	195	57	138	233	219	199	708

Figures in parentheses are rejected in calculations of mean, standard deviation, and normal range; also all data from Subject 24, who was obviously lipemic.

Table III

Plasma Lipids in Normal Men; Ages 70 to 101 Years

Plasma No.	Subject	Age	Lipids, mg. per 100 cc. plasma: Directly determined data: Total lipid C	Total lipid P	Total lipid N	Lipid amino N	Cholesterol: Total	Cholesterol: Free	Cholesterol: Esterified Equation 2	Calculated data: Cholesterol esters Equation 3	Phosphatides Equation 1	Neutral fats, etc. Equations 4, 5	Total lipids Equation 6
						Age class 70–74							
		yrs.											
45	Ha	71	415	5.7	7.8	2.9	249	111	138	235	135	54	535
46	Mo	72	915	12.8	16.1	5.3	327	87	240	406	302	407	1202
47	Vi	72	471	6 7	12 9	3.6	303	76	127	214	158	166	614
48	Ab	72	472	4.5	4 6	3.0	277	101	176	297	106	94	598
49	Sp	72	662	10.6	14 2	3.5	267	115	152	257	250	248	870
50	Ho	73	516	5 0	5.6	1.9	216	73	143	242	118	235	668
						Age class 75–79							
51	El	75	417	3.4	9.5	5.9	245	55	190	321	80	66	522
52	Is	76	513	8.7	19.1	2 3	235	74	161	272	205	120	671
53	Mi	77	545	8.4	6 1	3 7	207	100	107	181	198	239	718
54	Ob	77	716	13.6	14.7	3 9	353	(142)	211	357	321	128	948
						Age class 80–84							
55	Ph	80	823	12.5	16.3	5.2	189	104	85	144	295	560	1103
56	Kl	80	383	4.3	4.9	0.9	121	53	68	115	102	233	503
57	Ma	80	473	8.4	13.1	3.9	241	85	156	263	198	80	626
58	Ba	81	799	9 4	14.2	3.3	193	71	122	206	222	557	1056
59	Hu	82	588	8.4	11.2	1.6	238	71	167	282	198	217	768
60	In	82	778	12.7	18.8	5.5	156	86	70	118	298	542	1044
61	No	83	565	6.0	5.9	1.6	253	131	122	206	142	252	731
						Age class 85–91							
62	Ha	85	597	10.2	13.0	4.7	245	76	169	286	240	175	777
63	Me	85	648	16.6	17.2	4.5	261	86	175	296	392	103	877
64	Ad	89	531	9.2	9.6	3.3	214	64	150	254	217	165	700
65	St	91	676	6.7	11.8	3.9	253	84	169	286	159	351	880
66	Ja	91	445	8.2	12.6	4.9	202	82	120	203	192	107	584
						Age class over 100							
67*	Be	101	218	2.7	13.3	1.9	118	58	60	101	45	59	263

* All data from Subject 67 are excluded in calculation of mean, standard deviation, and normal range.

number of observations. Only when N is small is the difference between standard deviations calculated by the two formulæ important; *e.g.*, with $N = 20$ the difference is 2.8 per cent.

$\bar{x}$ = mean of all observed values of a given plasma constituent
x_i = an individual observation
N = number of observations
g = " " classes into which observations are divided. In the present case each age group is a class
$\bar{x}_j$ = mean of observed values within a given class
j = subscript used to denote a single class. j may have any value from 1 to g
n_j = number of observations in a single class
$\sigma_1 = \sqrt{\Sigma(x_i - \bar{x}_j)^2/(N - g)}$. This is a modified standard deviation indicating variance *within* classes (see Fisher (1932))
$\sigma_2 = \sqrt{\Sigma n_j(\bar{x}_j - \bar{x})^2/(g - 1)}$, a modified standard deviation indicating variance among classes (see Fisher (1932))

Rejection of Certain Data

In a collection of hundreds of intendedly normal data, some may deviate from the mean so unusually far that they are probably outside the group of observations which one would consider "normal;" *e.g.*, for plasma lipid content "normal" may be taken to mean a lipid concentration, determined during the postabsorption period without significant analytical error, in the plasma of a healthy subject, on an ordinary diet and free from unusual physiological influences. In a series of many lipid determinations taken with the intent to meet these conditions, some may fail to do so because of error in analysis, or because of undetected abnormality in a supposedly healthy subject, or because of some unusual physiological disturbance in a normal subject, analogous to the well known effect of emotion in raising blood sugar concentration of a normal subject to a "diabetic" level.

Among the data presented in this paper a few deviate so far from the general range that it appears more exact to exclude them than to include them in estimating normal means and ranges. Subject 24 (Table II) showed a total lipid content so far above the range of all the other subjects that it appears consistent with the probable truth to consider that he had a lipemia from some undetected metabolic abnormality. On the other hand, Subject 67 (Table III, 101 years old) showed a total lipid content much below

the range of all others. We have accordingly considered these two subjects as probably abnormal with respect to their lipid metabolism, and have omitted their data from the calculations of Tables IV, V, and VI, and from the graphs.

TABLE IV

Mean Values and Estimates of Fisher's Standard Deviation, s, for Directly Determined Lipid Values of Different Age Classes

The figures represent mg. of lipid per 100 cc. of plasma.

Age interval	No. of subjects	Total lipid C		Cholesterol						Lipid P		Lipid NH_2-N		Lipid total N	
				Total		Free		Esterified							
		Mean		Mean	*s*	Mean	*s*	Mean	*s*	Mean	*s*	Mean	*s*	Mean	*s*
yrs.															
20–39	10–7	537	146	214	82	85	16	129	68	6.6	2.9	3.3	1.4	10.2	5.7
40–44	4	519	100	220	26	83	18	137	14	7.9	2.7	2.9	2.1	10.7	3.1
45–49	5	472	75	253	42	74	13	179	31	6 2	3.0	2.4	1.6	10.8	3.1
50–54	5	546	140	220	67	73	17	146	52	7.6	2.3	3.1	1.3	8.7	3.6
55–59	8	681	271	246	68	78	15	168	53	6.4	3.0	2.3	1.4	10.1*	3.8*
60–64	6	535	170	246	71	90	12	157	70	8.8*	3.6*	3.8	2.6	10.2	5.7
65–69	4	524	84	222	39	79	32	143	52	9.0	1.6	4.4	1.0	17.6	4.7
70–74	6	575	186	257	44	94	18	163	41	7.6	3.4	3.4	1.1	10.2	4.8
75–79	4	548	125	260	64	77*	22*	167	46	8.5	4.2	4 0	1.5	12.4	5.7
80–84	7	630	173	199	49	86	25	113	40	8.8	3.1	3.1	1.8	12.1	5.1
85–89	3	592	59	240	24	75	11	165	13	12 0	4 0	4.2	0.7	13.3	3.9
90–91	2	560	164	228	36	83	1	144	35	7.4	1.1	4.4	0.7	12.2	5.7
Grand mean...		566	166	232	62	82	17	146	56	7.7	3 0	3.2	1.6	11.1	4.7

* Data from Subjects 24 and 67 are excluded from the calculations. Also, in calculating a mean or *s* value marked *, one figure is excluded, as indicated in Tables I, II, III.

In addition, of the some 390 directly determined lipid values of the other subjects, five single determinations are so definitely outside the ranges of other determinations of the same kind that their rejection seems justified. These observations are marked by figures enclosed in parentheses in Tables I to III, and by black squares on Figs. 1 to 8.

Non-Effect of Age on Concentrations of Lipids in Plasma of Healthy Men

Mere inspection of Figs. 1 to 8 and of the means and standard deviations of data from the different age classes in Table IV suffices to show that there is no regular important change in any lipid fraction with advancing years from youth to old age.

TABLE V

Observed σ Ratios in Analysis of Variance

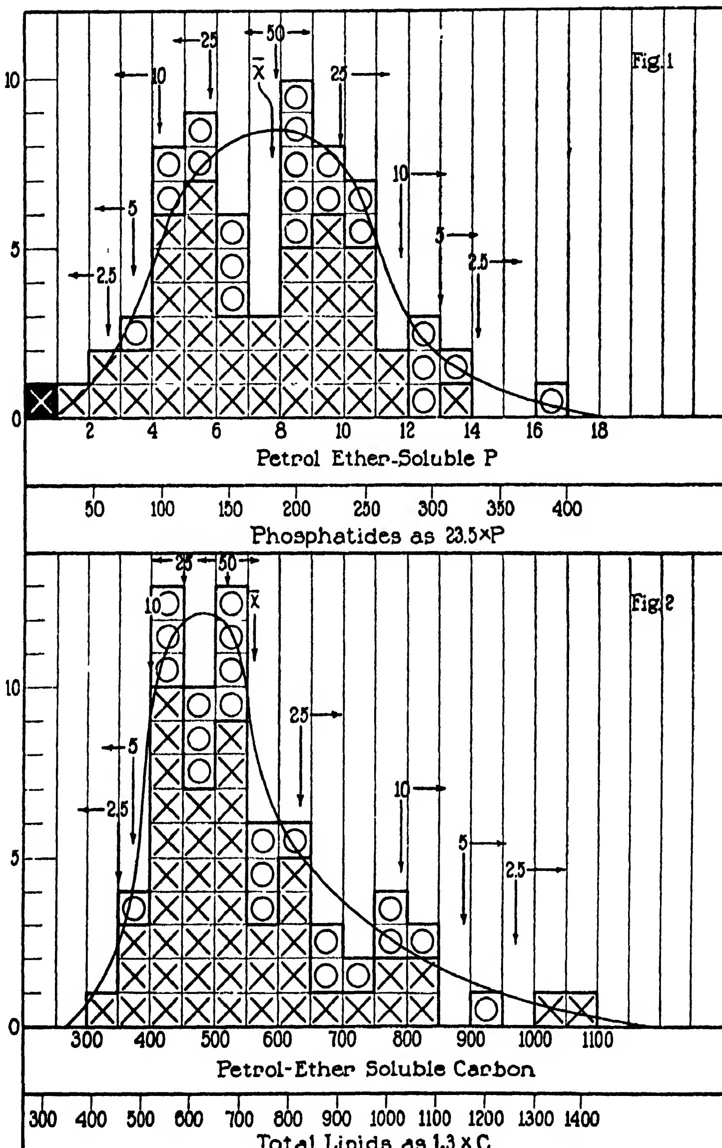
The ratios of estimates of σ are based, respectively, on the deviations of subclass (age group) means from the grand mean, and deviations of observed values from their subclass means

	Total lipid C	Total cholesterol	Free cholesterol	Esterified cholesterol	Lipid P	Lipid amino N	Lipid total N
No. of observations, N	66	65	66	65	66	67	67
No. of subclasses, g	13	13	13	13	13	13	13
σ_2/σ_1	1.01	0.76	0.79	1.10	1.05	0.93	0.96
Critical range*	1.393	1.394	1.393	1.394	1.393	1.391	1.391
	0.647	0.647	0.647	0.647	0.647	0.647	0.647

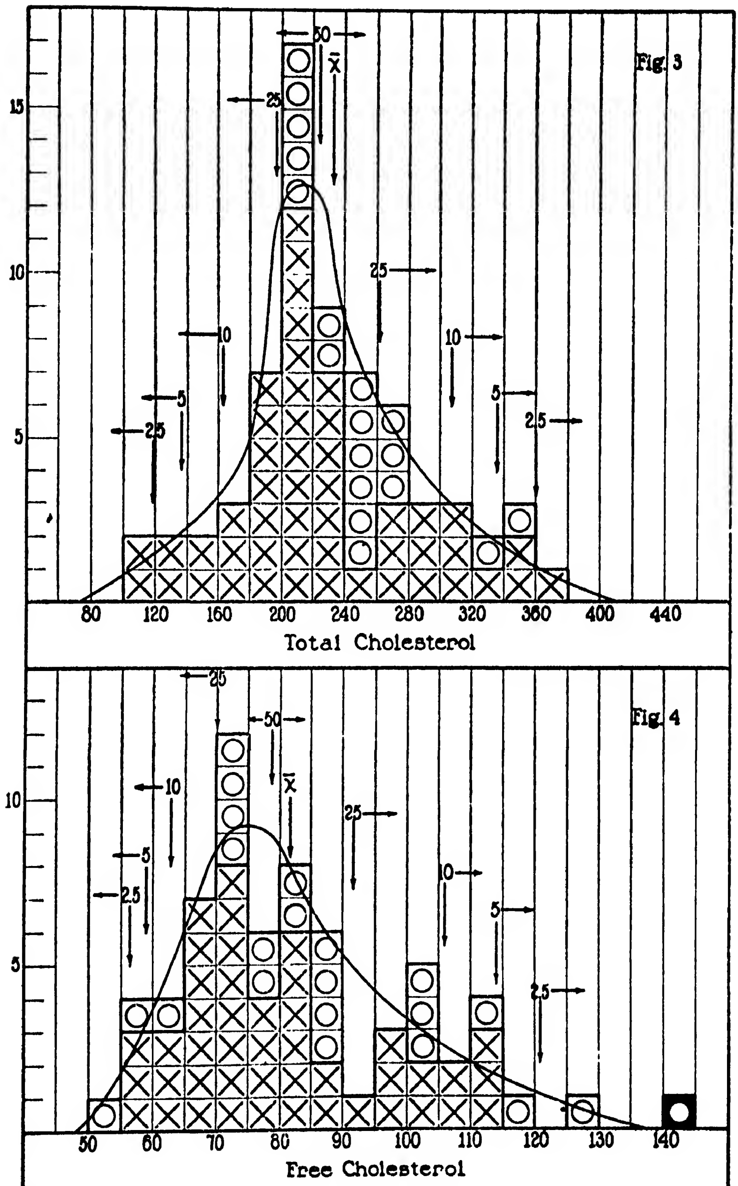
* In similar random sampling from a normal universe, the probability is 0.05 that the σ ratio would be above this range, and 0.90 that it would lie within the range. The upper bound of this range is obtained from its natural logarithm, Fisher's z, by entering his tables for *the 5 per cent point* with his $n_1 = g - 1$, and his $n_2 = N - g$. The lower bound of the range is found similarly by interchanging the values just given for Fisher's n_1 and n_2, and taking the natural antilogarithm of the value of z so found.

Statistical analysis indicates that there is, in fact, no more difference among different age classes than would be expected from the variability among individuals of similar age. The results of such analysis are given in Tables IV and V. In no case does the critical ratio lie between the ratio σ_2/σ_1 and unity. The critical ratio (c) is a number such that σ_2/σ_1 shall lie between c and $1/c$ nineteen times in twenty trials.

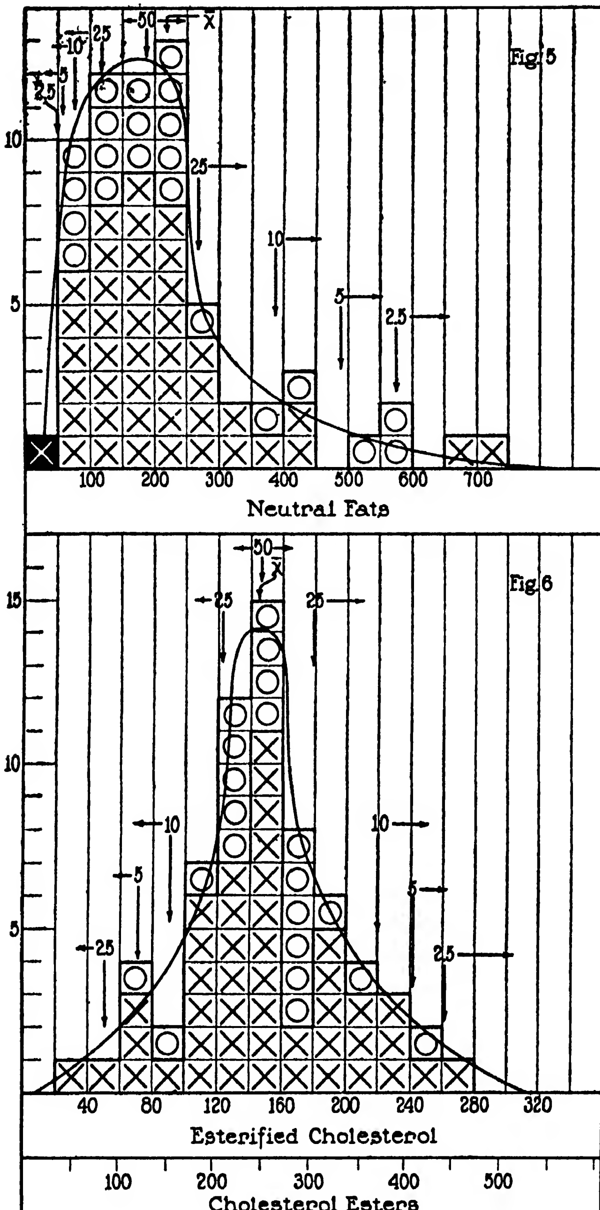
The value of c is obtained from Fisher's (1925, 1932) z tables by the formula, $z = |\log_e c|$.



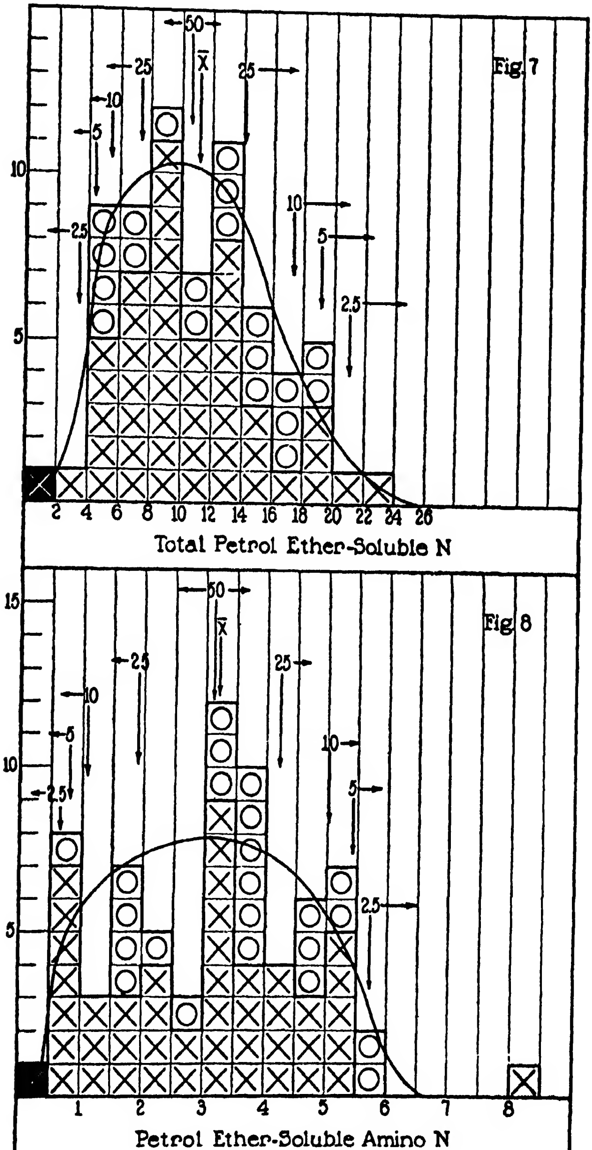
FIGS. 1 AND 2. Distribution diagrams for plasma phosphatides and total lipids. For explanation see text.



FIGS. 3 AND 4. Distribution diagrams for plasma total cholesterol and free cholesterol. For explanation see text.



FIGS. 5 AND 6. Distribution diagrams from plasma neutral fats and cholesterol esters. For explanation see text.



Figs. 7 and 8. Distribution diagrams of lipid total nitrogen and amino nitrogen. For explanation see text.

Estimation of Normal Lipid Ranges in Plasma of Adult Men

Since none of the substances determined shows any effect of changes in age over 20 years, the entire set of results, with the exclusion of the few discussed under "Rejection of certain data," may be used to estimate the normal concentration ranges of the lipid fractions in the plasma of men.

When data are distributed in accordance with the probability equation, one can calculate from the standard deviation the value of h for the range $(\bar{x} + h)$, $(\bar{x} - h)$, within which all except a given proportion, P, of observations may be expected to fall. For such calculations one may use Fisher's equation: $h = st\sqrt{(N+1)/N}$, where t is the t value given in the tables of "Student" (1925) and Fisher (1925, 1932) for given values of P, and N and s and N have the significance defined above under "Symbols." However, application of the probability equation to values such as plasma concentrations would lead to the impossible conclusion that the ranges extend to negative values. Furthermore, the frequency diagrams of some of the lipid values, notably total lipids and neutral fats (Figs. 2 and 5), are definitely and markedly so asymmetrical that it is evident that the probability curve is not even approximately followed; values above the mean extend so far that, if they went equally far below it, they would extend below zero. With these two lipid values, ranges coinciding fairly well with observed ones could be calculated when logarithms of the values, instead of the values themselves, were used. On the other hand, when the logarithmic procedure was applied to calculation of ranges of the lipids which showed more symmetrical frequency curves, the estimated ranges were obviously higher than the observed ones.

In view of the difficulty of finding a general application of the probability equation which would fit the different lipids, we have resorted to an empirical graphic analysis of the frequency diagrams. In each of Figs. 1 to 8 a smooth curve was drawn covering an area equal to that covered by the diagram, and equalizing the irregularities in its boundary. With a planimeter, areas at right and left ends of the diagram were then measured, such that they included 2.5, 5, 10, 25, and 50 per cent of the total area. The abscissa corresponding to the inner limit of each area is marked on the diagram with an arrow. It indicates, as nearly as can be estimated from our data, the value above which 2.5, 5, etc., per

cent of normal observations fall if the arrow is at the right, and below which these percentages of observations fall if it is at the left of the median line. The range between the two 25 per cent arrows includes 50 per cent of probable observations, and therefore marks the ± "probable deviation." This range probably does not deviate greatly from that which would be yielded by an infinite number of analyses. The location of the range limits nearest the extremes of the diagram is, of course, least certain.

The ranges thus obtained for the different lipids are presented in Table VI. Except in the case of the neutral fats, the ranges found to include 95 per cent of the observed values (with 2.5 per cent below and 2.5 above) coincide fairly well with the ranges calculated in accordance with the probability equation as $\bar{x} \pm 2.02\ s$, from the $\bar{x}$ and s values in Table IV. But when the theoretically calculated range is extended to $\bar{x} \pm 2.41\ s$, to include 98 per cent of probable observations, it runs into negative values on the lower side.

The point marked $\bar{x}$ on each of Figs. 1 to 8 is the mean. The point marked $\underset{\downarrow}{\leftarrow 50 \rightarrow}$ is the median. The blackened squares in Figs. 1, 4, 5, 7, and 8 indicate rejected observations, which were excluded in calculating the mean, median, and normal ranges.

Composition of the Plasma Lipid Mixture

In Fig. 9 and Table VII it is evident that as the total lipid concentrations in the plasma rise there is a tendency for each of the four constituent fractions to take part in the rise, but not to equal extents. Free cholesterol takes the least and neutral fat the greatest part in the changes of total lipid concentration.

The mean percentage of total cholesterol in the esterified form was 63.1, with a standard deviation of ±8.3. The percentage showed no significant change with the degree of lipemia: in the three groups of observations recorded in Table VII the values of the mean percentage and standard deviation were 61.3 ± 9.2, 65.8 ± 6.0, and 64.0 ± 8.5, respectively.

There appears to be an approximation to a reciprocal relation between cholesterol esters and neutral fats with regard to the proportion of each in the lipid mixture, as shown by Fig. 10. Cholesterol esters plus neutral fats comprise a fairly steady 65 ± 10

TABLE VI

Ranges of Postabsorptive Plasma Lipid Concentrations in Normal Men, Estimated by Integration of Smoothed Frequency Curves

The concentrations are expressed in mg. per 100 cc. of plasma.

Lipid substance	Per cent of observations: 2.5	5	10	25	Median concentration	Per cent of observations: 25	10	5	2.5
	Concentrations below which these percentages of observations fall					Concentrations above which these percentages of observations fall			
Directly determined data									
Total lipid C.	350	370	400	450	520	630	790	890	970
" " P.	2.6	3.4	4.2	5.8	7.9	9.9	11.8	13 0	14.2
" " N.	3.4	4.4	5.5	6.4	10.7	14.2	17.4	18.2	21.0
" " NH_2-N.	0.6	0.8	1.1	1.9	3.1	4.2	5.0	5.4	5.7
" cholesterol...	118	136	162	197	224	261	307	335	360
Free " ...	56	59	63	70	79	92	106	114	121
Calculated from direct data									
Esterified cholesterol.	51	72	91	123	148	180	220	242	261
Cholesterol esters.	86	122	154	208	250	304	372	410	440
Phosphatides.	60	80	95	135	185	230	275	305	335
Neutral fats.	50	60	80	120	190	270	390	490	580
Total lipids as 1.3 × C.	450	480	520	580	670	820	1030	1150	1260
Sum of free cholesterol, cholesterol esters, phosphatides, neutral fats.	250	320	390	530	700	900	1140	1320	1410

The difference between the minimal value, 250, in the bottom row and that, 450, in the row next above indicates that *minimal values for each of the four fractions summated in the bottom row are not likely to occur together in the same plasma.* (Minimal values in the table are those below which only 2.5 per cent of observed values are estimated to fall.) Otherwise the sum of the minimal values of these four fractions would approximate the observed minimal total lipid value, since these fractions, as calculated, make up the total lipids. Similarly, the observed maximal total lipid, 1260 mg., in the last column, is not so high as the sum of the maximum values of the four fractions, indicating that *maximum values for all four fractions are not likely to occur together.* As shown by Fig. 9, all four fractions tend in general to rise and fall together. However, independent variations among them are such that the chance for proportional falls or rises in all of them to occur at once in a given plasma is small.

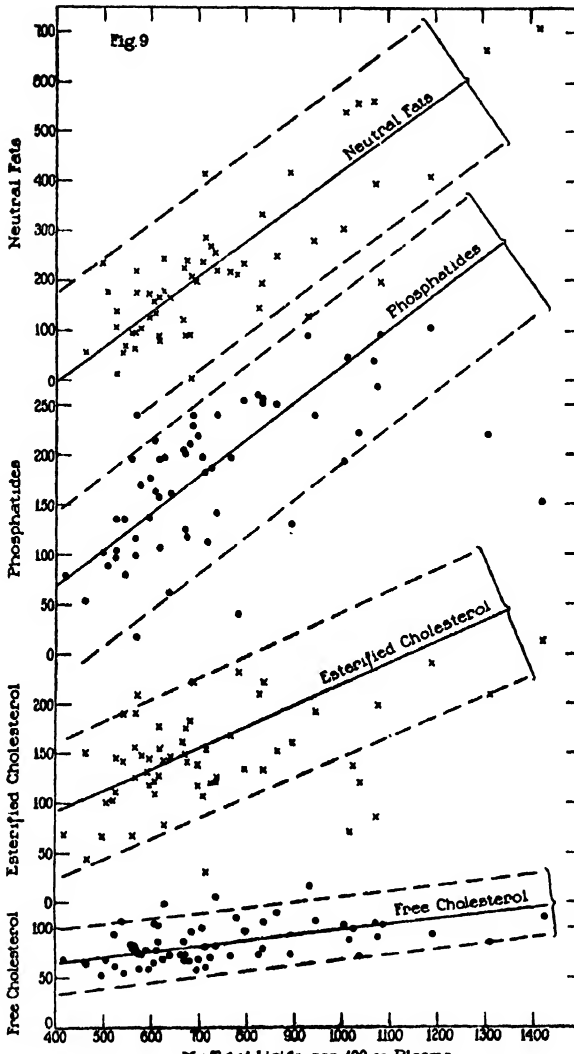


FIG. 9. Parts played by different lipid fractions in increase of total plasma lipids. Numbers represent mg. per 100 cc. of plasma. Note that a given vertical rise on the neutral fat scale indicates twice as much increase as a similar rise on the scale of any of the other three lipid fractions. It appears that increase of total lipids is likely to be due, in descending order of amounts, to neutral fats, phosphatides, cholesterol esters, and free cholesterol.

per cent of the total lipids, but the greater part of this percentage may consist of either of these two. Of course, rise in the percent-

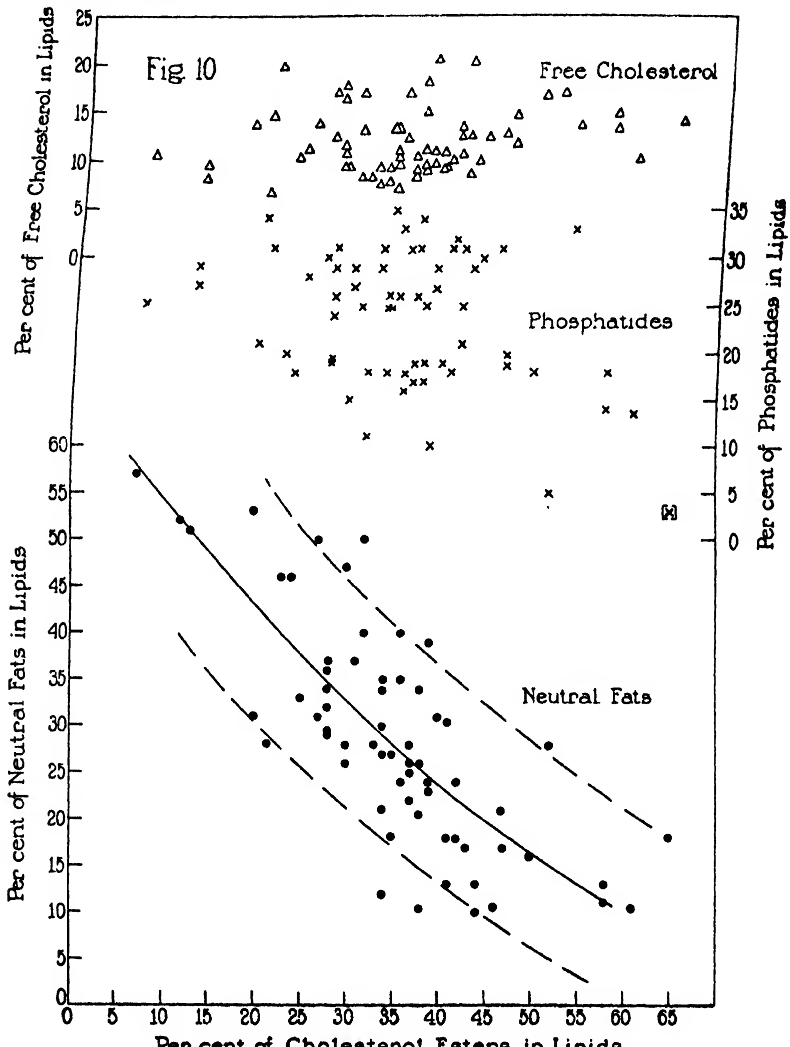


FIG. 10. Change of proportions of other lipid fractions with increase in proportion of cholesterol esters in plasma lipid mixtures.

age of any of the four fractions in the lipid mixture is necessarily accompanied by an equal fall in the sum of the percentages of the

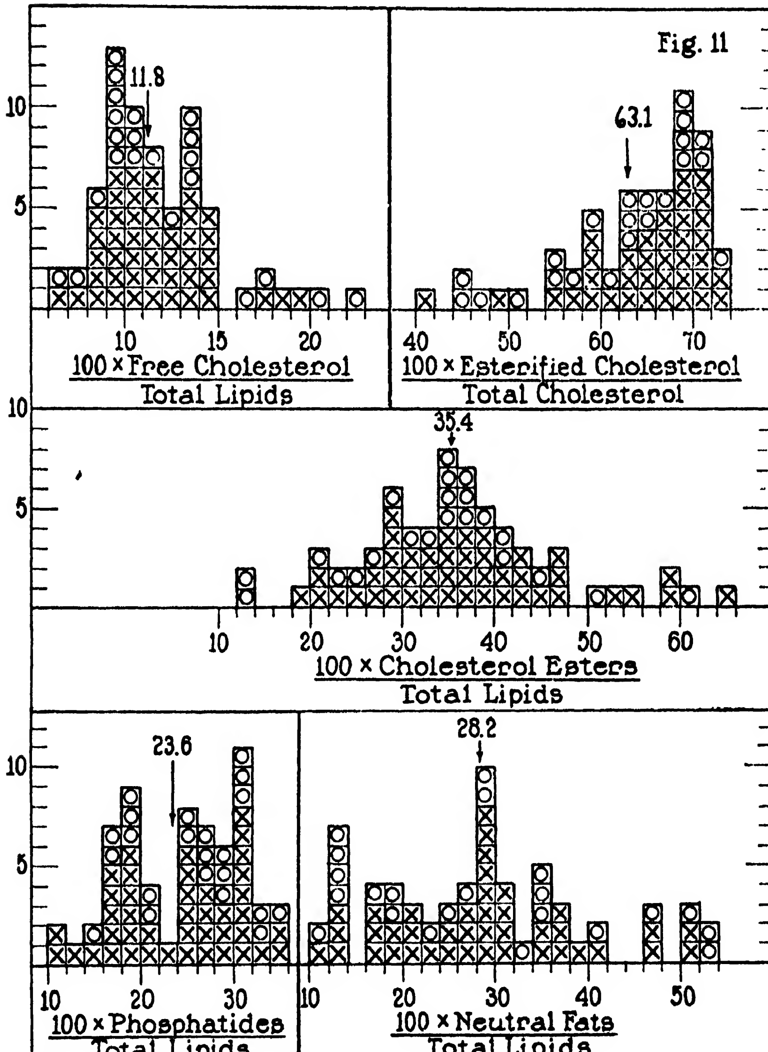


FIG. 11. Distribution diagrams indicating percentages of different lipid fractions in the total lipid mixture. The crossed areas represent data from men under 70, the circled areas data from men from 70 to 91. The number over the arrow on each diagram indicates the mean percentage of the lipid in the total lipid mixture.

other three. It appears, however, that as the cholesterol ester percentage in the lipid mixture deviates from the mean, the compensatory change in the other fractions is likely to occur chiefly in the neutral fat, and *vice versa.*

The distribution diagrams in Fig. 11 indicate that age of the subject has little effect on the composition of the plasma lipid mixture.

TABLE VII

Change of Lipid Composition with Varying Degree of Normal Lipemia

Range of total lipids	400–650			651–800			801–1400			All unrejected observations 400–1400		
No. of analyses	23–24			19–21			18–20			*Ca.* 64		
Lipid fraction	Mean concentration	Percentage in total lipids: Mean	Percentage in total lipids: s	Mean concentration	Percentage in total lipids: Mean	Percentage in total lipids: s	Mean concentration	Percentage in total lipids: Mean	Percentage in total lipids: s	Mean concentration	Percentage in total lipids: Mean	Percentage in total lipids: s
	mg. per 100 cc.	*per cent*		*mg. per 100 cc.*	*per cent*		*mg. per 100 cc.*	*per cent*		*mg. per 100 cc.*	*per cent*	
Free cholesterol	77	14.1	±3.0	79	11.2	±1.9	92	9.4	±1.9	82	11.8	±3 2
Cholesterol esters	217	38 3	±11 0	247	38 0	±8.1	299	30.0	±8.1	247	35.4	±10.3
Phosphatides	132	22.8	±6.3	173	23.9	±6.3	243	24.0	±5.4	181	23.6	±6.4
Neutral fats	131	23.3	±9.4	205	28.0	±8 0	355	34.0	±12.7	193	28.2	±11.3

s indicates standard deviation.

Nitrogenous Compounds in the Petroleum Ether Extract

If the nitrogen in the petroleum ether extract were derived entirely from lecithin and cephalin, the atomic N/P ratio would be 1, and the amino N/P ratio would fall below 1, parallel with the proportion of lecithin in the lecithin-cephalin mixture. However, Fig. 12 indicates that the N/P ratio is usually 2 to 5, and that the amino N/P ratio exceeds 1 in a considerable proportion of petroleum ether extracts. The mean atomic N/P ratio in our observations was 3.4, with a standard deviation of ±1.5; the mean atomic ratio of amino N to P was 1.00, with s = ±0.53. Kirk, Page, and

Van Slyke found evidence of a phosphatide with a N/P ratio of 2, but the proportion in the total phosphatide mixture was small.

The probable explanation of the high and extremely variable

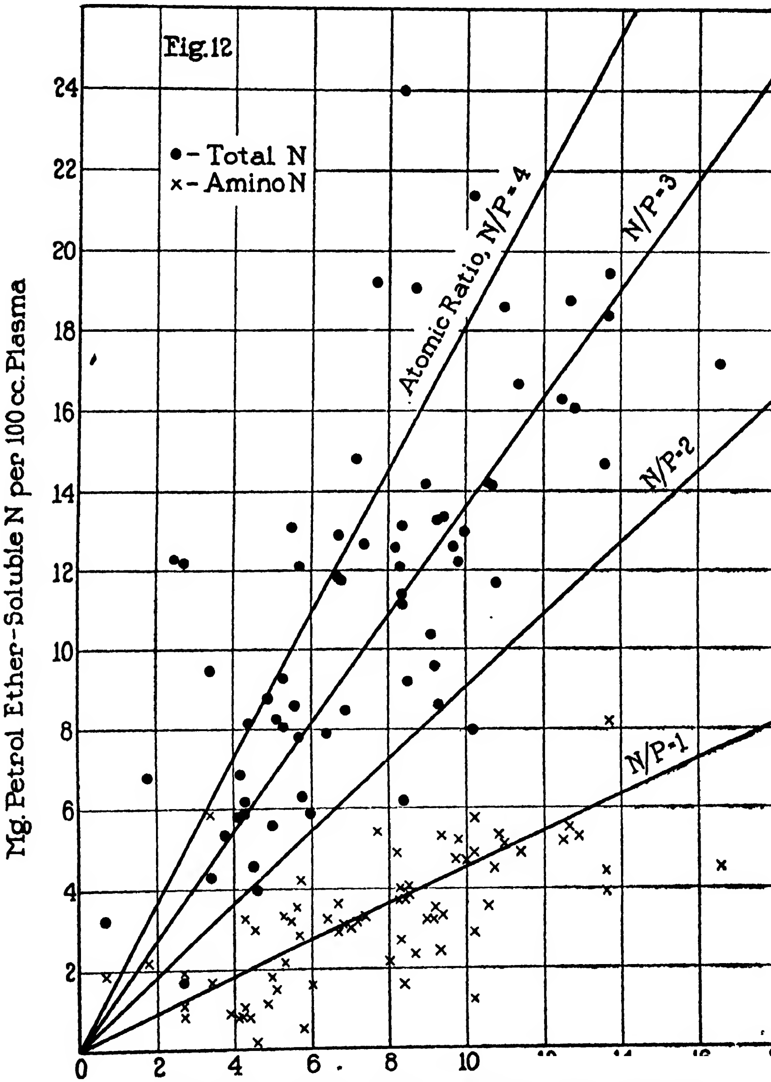


FIG. 12. Relation of phosphorus to total nitrogen and amino nitrogen in the petroleum ether-soluble material of plasma.

N/P ratios is that some plasma nitrogenous substances other than the phosphatides are extracted with alcohol-ether and redissolved by petroleum ether. The nature of these substances is at present uncertain. Page, Pasternak, and Burt (1930) found that about 45 per cent of the petroleum ether-soluble nitrogen in blood extracts was not precipitable with the phosphatides by $MgCl_2$ and acetone. An attempt to identify the non-phosphatide nitrogenous material has been begun by Van Slyke, Page, Kirk, and Farr (1935). It appears to be of relatively low molecular weight, as the carbon content is only a few times the nitrogen. It behaves like a base in being extracted from petroleum ether by acidified water. A biological test for choline was negative. The study is being continued.

Comparison of Present Values for Normal Plasma Lipids with Values Obtained by Other Methods

For cholesterol it appears that only data obtained with the digitonide method need be considered, for the inaccuracy of the colorimetric procedures is recognized (see criticism of the latter by Man and Peters (1933)). For the other lipids, recent methods have developed rapidly, and it appears desirable to use for comparison only the later modifications of Bloor's (1928) and Stoddard and Drury's (1929) methods, as developed by Man and Gildea (1932–33). As such we have taken the data in Table VIII.

The mean results of Man and Peters differ from ours probably by no more than might be expected, considering the wide range of variation in individuals on unrestricted diets, and considering the directions which errors of analysis are likely to take by their methods and by ours. Their results for phosphatides are calculated from the total phosphorus extracted by alcohol-ether, whereas ours are from the phosphorus which is extracted with alcohol-ether and then redissolved in petroleum ether. The added step in purification by resolution in petroleum ether may eliminate as much as 20 or 30 per cent of the alcohol-ether-soluble P, as exemplified by the results in Table IX. The difference between Man and Peters' mean results for phosphatides and ours is about what might be expected from this difference in technique.

On the other hand, one would expect the Man and Gildea methods used by Man and Peters to give somewhat lower results than ours for total lipids. Estimation of the total lipids from the

Table VIII

Results from Recent Literature for Lipids in Normal Human Plasma

The results are in mg. per 100 cc. of plasma. The figures after ± signs are standard deviations calculated as on p. 385.

Authors	No. and sex of subjects	Cholesterol		Phosphatides	Neutral fats	Total lipids	Methods of analysis
		Total	Free				
Gardner and Gainsborough (1927)	21 ♀	153 ±33	54 ±17				Gravimetric digitonide method before and after saponification
	22 ♂	169 ±41	50 ±21				
Man and Peters (1933)	12	207 ±29		222 ±29		659 ±80	Total cholesterol by gravimetric digitonide method after saponification. Phosphatide as total P extracted by alcohol-ether. Total fatty acids by NaOH titration after saponification
Boyd (1935)*	8 ♀	177	52	185	131	582	Free and total cholesterol by $H_2Cr_2O_7$ titration of Bloor on digitonide pptd. before and after saponification. Phosphatides by acetone-$MgCl_2$ pptn. and $H_2Cr_2O_7$ titration. Total fatty acids + total cholesterol by saponification, pptn., and $H_2Cr_2O_7$ titration. Neutral fat by difference. Total lipids by summation
Present writers	66 ♂	232 ±62	82 ±17	181 ±71	225 ±137	735 ±216	Free and total cholesterol by C in digitonide ppts. obtained before and after saponification. Phosphatides as total P soluble in petroleum ether. Total lipids from total C soluble in petroleum ether. Neutral fat by difference

* Boyd analyzed several plasma samples, drawn at different times of day, from each subject. The results of the separate analyses are not given, so that the standard deviations cannot be calculated.

Man-Gildea data is based in large degree on the value for total fatty acids, which are determined after isolation by saponification and precipitation. Any error would be likely to arise from losses in these operations, and hence to lead to low rather than high results. On the contrary, in the gasometric methods, the total lipids are estimated from the total carbon of the extractives soluble in petroleum ether. There is no occasion for low results in determination of this carbon. On the other hand, it includes small amounts of substances which are not fatty acid or cholesterol compounds (see the preceding discussion of nitrogenous compounds in the extract). Such substances would increase the total lipids above those obtained by the Man-Gildea method.

TABLE IX

*Comparison of Phosphorus in Alcohol-Ether Extract with That Left after Resolution in Petroleum Ether**

The figures indicate mg. per 100 cc. of plasma. Both plasmas were from subjects with lipemia.

	Plasma 1	Plasma 2
P in alcohol-ether extract	38.30	32.26
	38.43	32.49
" redissolved in petroleum ether	26.95	24.59
	26.59	23.95
Percentage of alcohol-ether-soluble P redissolved in petroleum ether	70	75

* Phosphorus determinations were made in duplicate by the method of Kirk (1934), applied to plasma extracts as described on p. 222 of Kirk, Page, and Van Slyke (1934).

Total lipids were not calculated by Man and Peters. We have estimated them from their data by the approximate formula:

$$\text{Total lipids} = \text{total fatty acids} + \text{total cholesterol} + 7.5 \text{ (lipid P)}$$

The formula is derived as follows: Combination of each mole of fatty acid in the lipids is accompanied by the elimination of 1 mole of water, equal in weight to 0.064 of the weight of stearic or oleic acid. Hence the total lipids could be calculated by adding to 0.946 $\times$ (total fatty acids) the free cholesterol and the glycerol, cholesterol, and nitrogenous phosphoglycerides which are combined with the fatty acids to form neutral fat, cholesterol esters, and phosphatides, respectively. The glycerol is not determined. If, however, we estimate that half the fatty acids are in the

form of neutral fats, correction for the glycerol would raise the coefficient of the fatty acids from 0.964 to nearly 1. The amounts of glycerylcholine phosphate and glycerylaminoethyl phosphate combined in lecithin and cephalin can be calculated as 6.8 P and 8.1 P. If the phosphatides consisted of a mixture of equal parts of lecithin and cephalin, the nitrogenous glycerylphosphates combined to form their mixture would be calculated as 7.5 × (lipid P). The factor 7.5 can be only approximate, but it appears improbable that deviations from it are sufficient to affect markedly the calculated total lipid values.

In Boyd's results, the relatively low values for total lipids and neutral fat are presumably in part attributable to the fact that in his technique, like that of Man and Peters, estimation of total lipids, and likewise the neutral fats, is based on the total fatty acid determination, probably subject to small minus errors, whereas our total lipid and neutral fat values depend on total lipid carbon, which is probably subject to small plus errors. To a large extent, however, it appears that the low total lipid and neutral fat values of Boyd may be due to differences in procedure for extraction of the lipids from plasma. These authors used the original Bloor technique, in which the plasma-alcohol-ether mixture is brought to a boil for only a moment, then cooled and filtered. Man and Peters and the writers, on the other hand, used the Bloor extraction as modified by Man and Gildea, who boil the plasma-alcohol-ether mixture for an hour under a reflux to complete the extraction. Man and Gildea found that the refluxing increased the yield of total fatty acids from 5 to 31 per cent above the yield obtained by the original Bloor extraction.

The cholesterol results of Boyd and of Gardner and Gainsborough (who also used the original Bloor extraction) are, like Boyd's total lipid values, markedly lower than the results of Man and Peters and of the writers. The most obvious common difference in procedure is that the authors who obtained the low results used the original Bloor extraction, while Man and Peters and the writers used the Man-Gildea modification. However, in analyses of two plasmas, each of which was subjected to both types of extraction, Kirk, Page, and Van Slyke (1934) (see their Table V) found no significant effect of this difference in mode of extraction on values obtained for either free or total cholesterol. It is possible that to some degree our higher results may be due to the use of a technique in which the cholesterol digitonide is precipitated, washed, and

determined in the same tube, without opportunity for loss in transfer to another vessel for oxidation (Bloor method used by Boyd) or for weighing (Gardner and Gainsborough). But such an explanation could hardly account for the difference between 50 and 80 mg. per cent of free cholesterol.

In order to ascertain whether our higher results might be due to incomplete washing out of other lipids from the cholesterol digitonide precipitate, we performed the following experiment.

In 5 cc. aliquots of petroleum ether extract from a lipemic plasma (with 1.7 per cent of total lipids) the free cholesterol was precipitated with digitonin as directed by Kirk, Page, and Van Slyke. The filtrate and washings from the precipitate were concentrated to dryness at 60°, and 0.1185 mg. of cholesterol in petroleum ether solution was added to each residue. The latter was redissolved in petroleum ether and again submitted to digitonide precipitation. The precipitate was equivalent, in duplicates, to 0.1049 and 0.1032 mg. of cholesterol, or 88 per cent of that added. The result was not too high, but too low. In control analyses of the standard cholesterol solution, the amounts regained were 0.1187 and 0.1218 mg., averaging 101.4 per cent of theoretical.

It appears that our washing procedure is adequate to remove other lipids from the cholesterol digitonide, and that our results for cholesterol determined in the presence of the other lipids are likely to be low rather than high. For the fact that our normal cholesterol values range so much higher than those of Boyd and of Gardner and Gainsborough, we therefore lack an explanation. We can find no source of error for our results, and none is obvious for theirs.

SUMMARY

The concentration ranges in plasma of normal men have been ascertained for the different plasma lipids determined by the gasometric methods of Kirk, Page, and Van Slyke.

Variations of age, from 20 to 90 years, have not been found to have a determinable influence on either the amount or the composition of the plasma lipids.

Part of the amino and non-amino nitrogen obtained in the petroleum ether extracts appears to be derived from substances other than phosphatides.

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THE EFFECT OF AGE ON THE PLASMA CALCIUM CONTENT OF MEN

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The object of the present work was to ascertain whether with advancing age, and the common tendency to calcification, changes occur in the plasma calcium concentration.

The subjects were all males, and were normal in so far as could be ascertained by clinical examination. They were part of the group who provided the plasmas for the accompanying study of plasma lipids (Page, Kirk, Lewis, Thompson, and Van Slyke (1935)). The plasma was obtained as there described. The calcium determinations were made in duplicate by the gasometric method of Van Slyke and Sendroy (1929).

The results given in Table I fail to reveal any effect of age changes up to the age of 85, beyond which the data are too few to permit interpretation. Statistical analysis of the results of age classes, by the method used in the accompanying paper (Page, Kirk, Lewis, Thompson, and Van Slyke (1935)), indicate that the variations between classes were no greater than would be expected by the variations within classes.

It appears that up to the age of 85 the plasma calcium content of the normal adult man undergoes no changes capable of detection by the data obtained.

TABLE I

Subject	Age	Plasma Ca	Mean for age class	Standard deviation from mean
	yrs.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Gr	40	10.0		
Dr	41	8.8		
To	41	9.7		
El	44	10.6		
McK	44	10.5	9.9	0.7
Cr	45	10.0		
Ed	47	9.1		
Co	47	10 8		
Ja	48	12.7		
Dy	49	9.7	10.4	1.4
Ho	50	13.0		
To	51	8.4		
Wi	51	10.5	10.6	2.3
Ne	55	8.3		
McM	55	12.0		
Na	56	10.7		
Bi	57	10.7		
Po	59	10.2	10.4	1.3
Ro	60	10.7		
Go	63	10.2		
Ed	63	9.6		
Ka	64	9.5		
Sh	64	9.0		
La	64	10.1	9.8	0.6
Pi	65	10.4		
Jo	66	11.1		
Wi	68	10.1		
Sm	69	9.3	10.2	0.8

Subject	Age	Plasma Ca	Mean for age class	Standard deviation from mean
	yrs.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Ha	71	9.8		
Mo	72	10.0		
Vi	72	10.7		
Ab	72	10.4		
Sp	72	10.2		
Ho	73	9.2	10.0	0.5
El	75	9.8		
Is	76	9.4		
Mi	77	10.9		
Ob	77	10.0	10 0	0.6
Ph	80	(8.0)		
Kl	80	10.3		
McK	80	9.6		
Ba	81	9.6		
Hu	82	10.1		
In	82	10.3		
No	83	10.5	9.8	0 9
Ha	85	9.6		
Ne	85	8.2		
Aa	89	10.0	9.3	1.0
St	91	9.2		
Be	101	8.1		

The figure in parentheses was excluded in calculating the mean and standard deviation.

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PLASMA LIPIDS IN CHRONIC HEMORRHAGIC NEPHRITIS

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(Received for publication, October 7, 1935)

That lipemia is frequent in renal disease was noted by Bright and his colleagues. Christison (1839), who in Edinburgh at once began to extend the studies begun by Bright in London, states that "In the early stage the serum is usually somewhat lactescent, and yields to sulfuric ether when agitated with it a small quantity of concrete oily matter, which seems to differ little from the fat of cellular tissue." Christison also noted that in the same stage of the disease the specific gravity and albumin content of the plasma were low. The "early stage" of Christison apparently includes what is now termed by Addis and others the chronic active stage. The two most striking serum changes, protein deficit and liquid excess, that occur in this stage, with its usual "nephrotic" character, were clearly recognized by the early observers. Evidence concerning the types of lipids and proteins affected, and the extent of their changes during the course of the disease, necessarily awaited later methods of analysis.

The earlier quantitative studies, on unclassified cases of Bright's disease, merely showed that samples of plasma from a considerable proportion of nephritic patients had high total lipid or cholesterol values (Chauffard, Laroche, and Grigaut, 12 cases (1911); Widal, Weill, and Laudat (1912); Denis, 30 cases (1917)). Gainsborough (1929) confirmed these results with regard to cholesterol by the gravimetric digitonin method, and determined both free and esterified cholesterol.

Evidence that high plasma cholesterol is characteristic of the nephrotic type or stage of Bright's disease was furnished by Epstein (1917), who published data from 66 renal and cardiac cases, of which 24 were of the nephrotic type. All cases of this type showed total colorimetric cholesterol values markedly above normal, while no other cases did.

Confirmative results from 2 cases of nephrotic and 21 of non-nephrotic Bright's disease were published by Port (1919), from 5 cases of nephrosis and 13 of glomerulonephritis by Hahn and Wolff (1921), and from 17 cases of nephrosis by Schwarz and Kohn (1922). Westphal (1924–5) found high serum total cholesterol (250 to 430 mgm.) in 2 cases of amyloid nephrosis. Murphy (1927) and Maxwell (1927–28; 1934) found increased plasma cholesterol in cases of nephritis with edema, but not as a rule in those without it. Lichtenstein and Epstein (1931) found both free and esterified cholesterol increased in nephrosis and in glomerular nephritis with edema. They also found that the proportion of cholesterol in the form of esters was increased to 80 to 90 per cent (compared with the usual normal of 60 to 70 per cent). With regard to the high proportion of esterified cholesterol their results are at variance with those of Gainsborough (1929) and the present writers.

Calvin and Goldberg (1931) found that, while cholesterolemia developed with nephritic edema, it sometimes continued for a year or more after the edema disappeared.

That, on the other hand, in the terminal stage of advanced nephritis, plasma total cholesterol is usually not above the normal range, and may fall to the lower part of this range during the last days or weeks, was found by Henes (1920) and by Ashe and Bruger (1933). In the intermediate chronic stage these authors found plasma cholesterol values either within or above the normal range. They believed that in an advanced case with nitrogen retention, a fall of plasma cholesterol was a grave prognostic sign.

Concerning the behavior of the lipids other than cholesterol the data have been conflicting. Bloor (1917), in a series of 23 unclassified renal cases, found that in most of them the total fatty acids were high, while cholesterol and phosphatides were normal, the high fatty acids apparently being due to high neutral fats. On the other hand, Daniels (1925) in 5 out of 7 nephrotic cases (chronic parenchymatous) found increases, not only in the total fatty acids, but also in the phosphatides and the cholesterol. Hoesch (1931) found high phosphatides in chronic nephritis. Knauer (1927) stated that in 15 cases of nephrosis he found increases of all 3 lipid fractions, but gave the figures for only one case. Ling and Liu (1928) studied 5 nephrotic cases, in

which they determined total fatty acids and unsaponifiable matter (mostly cholesterol), and found increased values for these two fractions which averaged respectively 310 and 170 per cent of the average normal.

Hiller, Linder, Lundsgaard, and Van Slyke (1924) reported abnormally high plasma total cholesterol and fatty acids in 3 cases of nephrosis and 3 of chronic active hematuric nephritis. Also, when 1 gram of butter per kilo was ingested these patients showed greater increase, both absolute and percentage, in plasma fatty acids than was shown by normal subjects. The respiratory quotient fell as much or more than in normal subjects after similar butter ingestion, indicating that the lipemic patients showed no delay or deficiency in their ability to burn fat. It was concluded that their lipemia might be due to a disturbance in the mechanism for transferring lipids from the blood to the tissue depots.

The cases of Daniels (1925) and of Hiller, Linder, Lundsgaard, and Van Slyke (1924) are described, but clinical data for characterization of the type and stage of renal disease in the cases of most of the other authors quoted are either scanty or lacking.

Ling and Liu (1928) and Gainsborough (1929) used gravimetric methods, but the analyses of other authors have been chiefly by colorimetric or nephelometric technique, presumably inferior in accuracy either to the methods now employed in Bloor's and Peters' laboratories or to the present gasometric procedures.

Concerning the question of the relative behavior of the different plasma lipids in the different types and stages of Bright's disease, it is evident that previous analytical data are conflicting, and that the clinical description of most of the reported cases has been too scanty to afford accurate characterization of the type and stage of the disease. We have therefore determined the different lipids in a series of cases under observation in this hospital.

Methods and Calculations

The gasometric methods of Kirk, Page, and Van Slyke (1934) were used for determining the lipids. The blood was drawn before breakfast and handled as described by these authors.

From the directly determined data in Tables I*A* and II*A* the

cholesterol esters, phosphatides, neutral fats, and total lipids, used in computing Tables I*B* and II*B*, were calculated as described by Page,

TABLE I*A*

Chronic Active Stage of Hemorrhagic Bright's Disease, with Nephrotic Syndrome

Cases are arranged in order of increasing severity, according to urea clearance (see Table I*C*). Lipid concentrations are in mgm. per 100 cc. plasma. Normal range for comparison is given at bottom of table.

Blood number‡	Total lipid C	Total lipid P	Total lipid N	Lipid amino N	Cholesterol	
					Total	Free
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1-a	822	9.3	19.5	6.9	245	107
1-b	2000	22.9	27.9	10.0	1168	244
2-a	1632	11 6	17.7	6.6	589	412
2-b	1783	22.1	24 4	8.1	630	216
2-c	1237	17.6	22.3	3.6	368	181
2-d	1200	12.6	15 3	7.1	555	199
3	810	5.0	7 2	3.1	158	123
4-a	1459	19.8	35.5	12.5	718	194
4-b	1183	16.2	11.3	4.7	666	183
4 c	939	17.3	14.2	4.6	535	238
5	930	10 9	13.6	4.5		142
6	1137	18.4	36 6	6.7	575	162
7	1669	22.1	20.7	6.6	800	263
Normal range*						
1/20 above	890	13.0	18.2	5.4	335	114
1/10 above	700	11.8	17.4	5.0	307	106
Median	520	7.9	10.7	3.1	224	79
1/10 below	400	4.2	5.5	1.1	162	63
1/20 below	370	3 4	4.4	0 8	136	59

‡ Bloods 1-a and 1-b are from Patient Number 1, etc.

* From Page, Kirk, Lewis, Thompson, and Van Slyke (1935). For more complete normal data see original. The figures on the line marked "1/20 above" are those at such levels that, according to the data of these authors, only 1 normal subject in 20 would show higher lipid concentrations. The figures on the succeeding lines are of similar significance.

Kirk, Lewis, Thompson, and Van Slyke (1935). The formulae used are:

Total lipids = Total lipid carbon × 1.3
Phosphatides = Lipid phosphorus × 23.5
Cholesterol esters = Esterified cholesterol × 1.69

Neutral fats = (Total lipids) − [(Free cholesterol) + (Cholesterol esters) + (Phosphatides).]

As pointed out by the above authors, the "neutral fat" fraction thus calculated contains some material besides the simple tri-glycerides.

TABLE *IB*

Composition of Plasma Lipid Mixture

Blood number	Percentage of total lipids as: Free cholesterol	Cholesterol esters	Phosphatides	Neutral fat + unidentified lipids	Percentage of total cholesterol esterified	Total N / P atomic ratio	Amino N / P atomic ratio
	per cent	*per cent*	*per cent*	*per cent*	*per cent*		
1-a	10	22	20	48	56	4 6	1.64
1-b	9	62	21	7	79	2.7	0.97
2-a	21	12	13	54	25	3.4	1.26
2-b	9	30	22	38	66	2 4	0.81
2-c	11	19	25	44	51	2.8	0.45
2-d	13	39	19	29	64	2.7	1.25
3	12	6	11	71	22	3.2	1.37
4-a	10	47	25	17	73	4.0	1.40
4-b	12	54	25	9	73	1.5	0.64
4-c	20	41	33	5	55	1.8	0.59
5	12		21			2.8	1.50
6	11	48	29	12	72	4.4	0.81
7	12	42	24	21	67	2.1	0.66
Mean	12	36	22	30	59	3.0	0.99
Standard deviation	±3.8	±17	±5.9	±21	±18	±1 0	±0.43
Normal* mean	11.8	35.4	23 6	28.2	63.1	3.4	1.00
Normal* standard deviation	±3.8	±10 3	±6.4	±11.3	±8.3	±1.5	±0.53

* Page, Kirk, Lewis, Thompson, and Van Slyke (1935).

Cases Studied

The plasma lipids have been determined once or more in each of 13 cases of hemorrhagic Bright's disease, of which 7 were in the chronic active, and 6 in the terminal stage. In classifying the cases we have followed the usage of Van Slyke, Stillman, Möller, et al. (1930). The chronic active cases are classified as those which have passed the initial stage and apparently become chronic, but in which the renal function

TABLE IC

Clinical Data on Patients Reported in Tables IA and IB

Series and blood number	Hospital number	Age and sex	Date	Blood pressure	Urine proteins per 24 hours	Blood urea N per 100 cc.	Urea clearance	Hemo-globin	Proteins per 100 cc. plasma			Edema	Red blood cells in 12-hour urine
									Globulin	Albumin	Total		
		years		*mm. Hg*	*grams*	*mgm.*	*per cent of average normal*	*per cent**	*grams*	*grams*	*grams*		*millions*
1-a	7049	27 M	March 19, 1934	114/66	15	20	78	82	2.0	1.7	3.7	+++	0
1-b	7049	27 M	November 23, 1933	134/84	15	19	91	85	3.3	1.4	4.7	++++	1
2-a	9246	20 F	October 7, 1933	138/90	16	7	77	88	2.8	1.4	4.2	+++	1.7
2-b	9246	20 F	December 1, 1933	148/98	20	9	68	91			4.0	++	
2-c	9246	20 F	January 26, 1934	150/102	12	15	56	91			4.6	+	
2-d	9246	20 F	May 24, 1934	146/90	9	21	45	86	2.4	2.0	4.4	0	
3	8658	19 F	February 22, 1934	168/106	9	9	54	67			4.1	+	15
4-a	9279	22 F	December 5, 1933	138/92	11	40	36	75	2.8	2.0	4.8	+++	35
4-b	9279	22 F	March 16, 1934	155/96	14	32	34	75	3.0	2 2	5.2	+	111
4-c	9279	22 F	June 26, 1934	160/108	7	27	28	70			5.2	+	7
5	9040	42 M	April 13, 1934	154/88	5	13	30	76	2.3	2.2	4.5	++	1.3
6	9172	41 F	April 2, 1934	120/72	9	44	27	66			6.1	+	0
7	8936	24 F	February 28, 1934	130/94	10	39	17–24	69	2.7	1.63	4.3	+++	0

* 100 per cent hemoglobin ≈ 20.7 volumes per cent O_2 capacity in men, 19.0 in women (Peters and Van Slyke (1931), p. 544).

has not fallen far enough to depress the urea clearance consistently below 20 per cent of the average normal value. The terminal cases are those in which the clearance has fallen permanently below 20 per cent of normal. As pointed out by Van Slyke, Stillman, Möller, et al., the chronic active or intermediate stage is usually dominated by the signs of the nephrotic syndrome, viz., edema, heavy proteinuria, and plasma protein deficit; but with passage into the terminal stage these signs to a varying degree recede, and hypertension, cardiac enlargement, and eye-ground changes are likely to become prominent.

The cases in Tables I*A*, I*B*, and I*C* are fairly characteristic of the chronic active stage. The nephrotic syndrome was prominent in all except Number 6. Cases 1 and 2 could be mistaken for pure nephrosis, were it not for their histories.

In Tables II*A*, II*B*, and II*C* are given analytical and clinical data from six cases representing different degrees of progress in the terminal stage.

DISCUSSION OF RESULTS

Plasma Lipids in the Chronic Active Stage of Hemorrhagic Nephritis

Lipid concentrations. Table I*A* shows that the total lipids in all the cases examined were between 1 and 2.6 grams per 100 cc. of plasma. Since 1 gram per 100 cc. is reached by only about 1 normal plasma out of 10, it is evident that definite lipemia is the rule in this stage of nephritis, with total lipids varying upwards from the highest zone of the normal range.

Composition of the lipid mixture. The increase as a rule affects all the observed lipid fractions, including the petroleum-ether soluble, "lipid" nitrogen. Table I*B* shows that in its composition the lipid mixture in these nephritic plasmas showed no characteristic difference from the mixture in normal plasma. The reciprocal variation of cholesterol esters and neutral fats noted in normal plasma (Page, Kirk, Lewis, Thompson, and Van Slyke (1935), p. 630) is more marked in this nephritic series, as indicated by the high standard deviations for cholesterol esters and neutral fats in Table I*B*. The number of cases is not sufficient, however, to show whether the apparent increased variance is characteristic of lipemic nephritis, or is merely due to the chance occurrence, among the 13 plasmas reported, of 3 or 4 showing

unusual deviations of the cholesterol ester:neutral fat ratio from the mean.

The results in Table I*B*, like thosé of Gainsborough (1929), fail to show any tendency for the ratio, esterified cholesterol: total cholesterol, to increase in the lipemia of nephritis.

One behavior, noted by Page, Kirk, Lewis, Thompson, and Van Slyke (1935) in normal plasmas with unusually high lipid contents, is not apparent in the nephritic plasmas of Table I. In normal plasmas with lipid concentrations in the upper range these authors observed a tendency for the "neutral fats" (which include some unidentified petroleum-ether soluble material) to constitute a greater, and the cholesterol, both free and esterified, a lesser percentage of the lipid mixture than when the total lipid concentration was in the middle or lower normal ranges. If whatever factors caused this behavior in normal subjects acted in entirely the same way in nephritis, one would expect a higher proportion of neutral fats, and a lower proportion of free cholesterol and cholesterol esters, than the average in normal plasma. The limited number of cases reported in Table I fail to show such a tendency with any consistency.

TABLE II*A*

*Plasma Lipid Concentrations in Terminal Stage of Hemorrhagic Bright's Disease**

Cases are arranged in order of increasing severity, according to diminishing urea clearance.

Blood number	Total lipid C	Total lipid P	Total lipid N	Lipid amino N	Cholesterol	
					Total	Free
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
8	835				252	70
9	850	9.6	14.0	4.6	395	134
10	763	9.6	46.7	9.2	361	111
11-a	849	8.1	30.6	7.3	296	105
11-b	578	6.0	24.6	6.7	328	96
12	488	5.8	25.6	6.3	241	96
13-a	360	3.2	26.6	6.9	139	56
13-b	321	3.0	7.5	1.2	152	56
13-c	330	1.9	5.9	2.1	122	56

* For normal ranges, see bottom of Table I*A*.

While our data fail to show any consistent abnormalities in the

composition of the lipid mixture of nephrotic plasma, Ling and Liu (1928) did find an abnormality in a property not determined in our analyses, viz., the degree of unsaturation. They found that the iodine number of the fatty acids in 5 nephrotic cases was only 59 to 97, compared with the normal average of 157. The tendency for the iodine number to fall as lipemia increases, however, seemed not to be limited to lipemia of nephrotic origin. In 5 diabetics with lipemia Ling and Liu found iodine numbers as low (45 to 98) as in nephrosis.

Relation of plasma lipid and plasma protein concentrations. Both plasma protein deficit and lipemia are characteristic of the nephrotic

TABLE II*B*

*Composition of Plasma Lipid Mixture**

Blood number	Percentage of total lipids as				Percentage of total cholesterol esterified	Total N / P atomic ratio	Amino N / P atomic ratio
	Free cholesterol	Cholesterol esters	Phosphatides	Neutral fat			
	per cent	*per cent*	*per cent*	*per cent*	*per cent*		
8	6	28			72		
9	12	40	21	27	66	3.2	1.1
10	11	43	23	23	69	10.7	2.1
11-a	10	29	17	44	65	8.4	2.0
11-b	13	53	19	14	71	9.1	2.5
12	16	40	20	24	60	9.7	2.4
13-a	12	30	16	42	60	18.3	4.7
13-b	13	39	17	29	63	5.5	0.9
13-c	13	26	11	50	54	6.8	2.4
Mean	12	35	18	32	64	9.0	2.5
Standard deviation	±2.7	±8.9	±3.7	±12 4	±5.9	±4.5	±1.2

* For normal means and standard deviations see bottom of Table I*B*.

syndrome, which is common in chronic active hemorrhagic nephritis (Van Slyke, Stillman et al. (1930)), and a fall in the proteins may accompany a simultaneous rise in lipids. In the individual cases, 1, 2, and 4, in which repeated determinations were obtained over periods during which the plasma proteins showed definite rises, the lipids showed in each patient an accompanying decrease. However, when the entire group of Tables I*A*, I*B*, and I*C* is considered, it becomes evident that there is no close proportionality between the ex-

TABLE IIC

Terminal Stage of Hemorrhagic Bright's Disease

Clinical data on subjects in Tables II*A* and II*B*

Series and blood number	Hospital number	Age and sex	Date‡	Blood pressure	Urine proteins per 24 hours	Blood urea N per 100 cc.	Urea clearance	Hemoglobin*	Proteins per 100 cc. plasma			Edema	Red blood cells in 12-hour urine
									Globulin	Albumin	Total		
		years		*mm. Hg*	*grams*	*mgm.*	*per cent*	*per cent*	*grams*	*grams*	*grams*		*millions*
8	8742	24 F	October 3, 1933	216/166	2	90	18	96				+	++
9	8883	28 F	March 13, 1934	144/84	9	72	17	69	2.7	2.6	5.3	+	3.7
10	9231	30 F	March 12, 1934	220/114	3	58	12	52	2.5	3.4	5.9	+	1.9
11-a	9270	49 F	December 1, 1933	128/80	2	51	7	78	3.3	3.0	6.3	0	0.8
11-b	9270	49 F	March 19, 1934	160/92	1	43	10	76			5.7	0	0.1
12	8916	16 F	January 31, 1934	160/104	2	145	6	27	2.4	3.2	5.6	++++	++
13-a	8982	21 M	February 14, 1934	168/96	17	146	4	31			5.8	+++	++
13-b	8982	21 M	February 28, 1934	200/114	15	160	6					+++	++
13-c	8982	21 M	March 8, 1934	196/116	12	238	3	32				+++	++

* 100 per cent hemoglobin ≈ 20.7 volumes per cent O_2 capacity in men, 19.0 in women (Peters and Van Slyke (1931), p. 544).

‡ Patient numbers 10, 11, 12, and 13 have died on the following dates: Number 10, September 9, 1934; Number 11, April 25, 1935; Number 12, February 9, 1934; Number 13, April 17, 1934.

tents of lipemia and plasma protein deficit. Thus Case 3 showed the least lipemia, but nearly the greatest deficit in plasma proteins. Absence of consistent parallelism between lipemia and plasma protein deficit was also noted by Daniels (1925).

Plasma Lipids in the Terminal Stage of Hemorrhagic Nephritis

Lipid concentrations. The cases in Table II may be divided into two groups, Cases 8, 9, 10, and 11, which were not yet in uremia, and Cases 12 and 13 which were advanced to definite uremic symptoms. Cases 8, 9, 10, and the first observation on 11, show total lipid values in the range covered by the upper fifth of normal plasmas, and 3 of the 4 were in the range of the upper tenth of normal. In these cases there still existed a definite tendency towards lipemia, although not so marked as in the less advanced cases of Table I. The transition from the nephrotic syndrome of the chronic active stage to the vascular-uremic syndrome of the terminal stage is not sharp, and it is evident that some tendency to lipemia frequently persists, like other nephrotic signs, into the terminal stage.

However, the two cases, Numbers 12 and 13, which had advanced to uremia, showed no tendency towards lipemia. In fact all three observations on Case 13 showed *lower* plasma lipid content than was observed by Page, Kirk, Lewis, Thompson, and Van Slyke (1935) in any of their 60 normal subjects.

Composition of the lipid mixture. Table II*B* shows no consistent deviation from the normal, except in the nitrogen. The N/P and Amino N/P ratios average respectively 2.6 and 2.5 times higher than in normal plasma. Even more than in normal plasma the nitrogen soluble in petroleum ether exceeds the values which could be attributed to the phosphatides (Van Slyke, Page, Kirk, and Farr (1935)). In Cases 10 and 11 the lipid nitrogen approaches the urea nitrogen in concentration. There is evidently at times in the terminal stage of nephritis a marked tendency for accumulation of some unknown, petroleum-ether soluble nitrogenous product or products. The accumulation, although it may sometimes nearly equal, does not at all parallel that of urea. It is striking that during the month when Case 13 was under observation for plasma lipids, the lipid nitrogen fell from 26 mgm. to the low normal level of 6 mgm., while blood urea nitrogen

rose from 148 to 238 mgm. The patient died a month after the last observation. *It* appears that the plasma lipid nitrogen, which is usually elevated in about the same proportion as the other lipids in the chronic active stage of hemorrhagic nephritis, may remain high in the terminal stage for some time after the other lipids have fallen, but that eventually the lipid nitrogen also may fall.

Calculation of Total Lipids in Lipemic Plasma

Page, Kirk, Lewis, Thompson, and Van Slyke (1935) have shown that, in normal plasma, total lipids can be calculated by the approximate formula, *Total lipids* = 1.3 × *Total lipid carbon*, with practically the same results obtained by calculating the total lipids as the sum of phosphatides, free cholesterol, cholesterol esters, and neutral fats. The latter calculation is theoretically the more accurate, but the difference between total lipids calculated in the two respective ways was found in normal plasma to average less than 1 per cent of the values obtained. The accuracy of the $1.3 \times C$ formula is a matter of some importance, because its use enables one to estimate the total lipids from a single carbon determination on the petroleum ether extract.

In order to obtain data on the accuracy of this formula when applied to lipids in pathological, and particularly in lipemic, plasma, we have calculated the total lipids in both ways from the data in Tables I*A* and II*A*. The 2 methods of calculation yield results agreeing in the pathological plasmas as closely as they were found to agree in normal plasma by Page, Kirk, Lewis, Thompson, and Van Slyke. It appears that one may use the $1.3 \times C$ formula for total lipids with fair assurance that the calculation error is practically within the limit of the analytical error, in pathological as well as normal plasma.

SUMMARY

The total lipids, in nephritic as well as in normal plasma, can be estimated from the total lipid carbon as $1.3 \times C$; the error involved in the use of the approximate factor 1.3 averages less than 1 per cent.

Single or repeated determinations of the different lipid fractions determinable by the gasometric method of Kirk, Page, and Van Slyke have been made on the plasmas of 13 patients with chronic hemor-

rhagic nephritis. The degree of renal damage varied from slight, with nearly normal urea clearance, to almost complete destruction, with only 3 per cent of normal clearance.

The results fit the following picture of the course of plasma lipid changes during the disease: In the chronic active stage (urea clearance over 20 per cent normal) there is a tendency to lipemia, with plasma lipids near or above the upper limit of normal levels; in the 7 cases reported in this stage, total lipids varied from 1 to 2.6 grams per 100 cc. of plasma, while normal subjects average 0.7 and seldom exceed 1.1. As the disease passes into the terminal stage (urea clearance under 20 per cent) the lipemia is likely to decrease, and before exitus the plasma lipid content may fall below normal. A similar course of total cholesterol change has been noted by Ashe and Bruger and by Henes. The present results indicate that it is followed also by the other lipids.

The individual lipid constituents, free cholesterol, cholesterol esters, phosphatides, and the neutral fat fraction, rise and fall together. The percentage of each in the lipid mixture, although variable, shows no characteristic trend of deviation from the percentage in normal plasma.

The amount of nitrogen soluble in petroleum ether also rises parallel with the other lipid constituents in the chronic active stage, but may not fall with them in the terminal stage. In consequence, the proportion of both total and amino nitrogen in the lipid mixture in the terminal stage averaged, in our cases, two to three times the proportion in normal plasma, or in the plasma from patients in the chronic active stage. The high atomic N/P ratio, from 3 to 18, in the terminal lipids is evidence that the nitrogen was chiefly in the form of petroleum-ether soluble substances other than the phosphatides.

The results, like those of Daniels, show no parallelism in severity between lipemia and plasma protein deficit, although both accompany the nephrotic syndrome.

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PLASMA LIPIDS IN ESSENTIAL HYPERTENSION

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Because of the frequent presence of cholesterol in the arterial lesions which develop during hypertension, the possibility was pointed out by Lemoine (1911) that hypercholesteremia might be a predisposing factor to atheromatosis. That it can be so in rabbits has been repeatedly demonstrated by administering cholesterol by mouth, with resultant production of hypercholesteremia and of atheromatous arterial lesions resembling those found in man (for literature see Wacker and Fahrig (1932)). The application of these results to other animals is made doubtful, however, by the fact that it is impossible to produce atheromatous lesions by feeding cholesterol to dogs or cats (Anitschkow (1925); Yuasa (1928)). Most investigators have failed to find hypertension in rabbits with arterial lesions produced by cholesterol (see review by Dominguez (1927)).

The first attempt to collect from human cases data bearing on the question appears to have been made by Lemoine (1911), who studied by a semi-quantitative color test the cholesterol content of the serum in clinical hypertension, and found it abnormally high. Confirmatory results from cases of hypertension, only part of which were "essential," were reported by Pribram and Klein (1924) (47 cases, 76 per cent with high plasma cholesterol), Westphal (1924–5) (80 cases, 71 per cent high), and Gelman (1927) (37 cases, 68 per cent high). Wacker and Fahrig (1932) reported 12 cases of essential hypertension, with 18 control analyses of subjects with normal blood pressure. The normal cases averaged 152 mgm. of total cholesterol per 100 cc., with 180 as the maximum, while the cases of hypertension averaged 207 mgm., and all but 3 exceeded 180.

On the other hand, Loewenstein (1928) reported 50 cases of hypertension, of which 4 were apparently nephritic and the rest essential;

of the 50 he found only one with serum total cholesterol above 180 mgm. per 100 cc., which he considered the maximum normal value by the colorimetric method used. Weinstein and Weiss (1931) found high total cholesterol in only 5 of 37 cases of uncomplicated essential hypertension. Kirchgessner (1934) reported that most hypertensive subjects, in a series of 49, showed high values for free cholesterol, although total cholesterol was usually normal. Bürger and Möbius (1934), in a paper appearing after our data were collected, found by the digitonin method entirely normal plasma concentrations of both free and total cholesterol in 20 cases of essential hypertension.

Alvarez and Neuschloss (1931), like Loewenstein, found normal total cholesterol values (colorimetric), in hypertension cases. They studied also, however, the ability of the serum to dissolve additional free cholesterol, and found that the sera of 21 out of 25 hypertension cases were supersaturated with cholesterol. Medvei (1932) attempted to confirm these results with regard to saturation, however, and was entirely unable to do so; he found the serum approximately saturated with cholesterol in both normal subjects and patients with hypertension. Only 2 of Alvarez and Neuschloss' 25 cases, and perhaps 8 of Medvei's 30, appear to be essential hypertension.

The available data show no agreement on the simple questions, (1) whether total cholesterol is high in the serum of patients with hypertension; and (2) whether the serum of such patients is supersaturated with free cholesterol. Only Kirchgessner's data, mostly colorimetric, bear on the question whether the ratio of free to esterified cholesterol is normal.

Data on plasma lipids other than cholesterol in hypertension are scant and contradictory. Wacker and Fahrig (1932) in the paper already quoted, found that phosphatides, neutral fats, free cholesterol, and esterified cholesterol all showed a similar tendency to a moderate increase in hypertension. Hoesch (1931) on the other hand found lipid phosphorus normal in essential hypertension (7 cases).

In view of the disagreement of results in the literature, we have determined, by the gasometric methods of Kirk, Page, and Van Slyke (1934) the plasma lipids of 16 patients in whom the diagnosis of essential hypertension appeared justified. Data from 2 patients with malignant hypertension also were obtained.

Methods and calculations are as described by the writers in the preceding paper.

TABLE I

Essential Hypertension

Subject			Clinical data					Lipids per 100 cc. plasma					
Number	Hospital number	Age and sex	Blood pressure	Urine proteins per 24 hours	Blood urea nitrogen per 100 cc.	Urea clearance	Hemoglobin per cent of normal for age and sex†	Total lipid C	Total lipid P	Total lipid N	Lipid amino N	Cholesterol Total	Cholesterol Free
		years	*mm. Hg*	*grams*	*mgm.*	*per cent of average normal*	*per cent*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	8846	40 M	220/138	0.4	24	76	86	480	3.5	12.7	2.6	163	67
2	8855	26 F	270/140	1.5	15	56	100	351	7.3	9.6	1.5	243	108
3	8990	22 F	173/130	0	13	86	100	479	5.5	6.1	2.2	192	73
4	8527	32 M	188/116	0	17	159	100	843	12.1	17.3	5.0	193	106
5	8311	68 M	171/68	0	13	82	108	524	6.4	9.9	3.2		87
6	9223	24 F	208/130	0	12	94	108	439	5.7	8.9	2.5	241	77
7	8947	28 M	175/106	0	7	119	97	608	7.3	13.5	3.3	119	88
8	8999	46 M	218/116	0	13	74		666	8.9	16.8	2.6	284	103
9	4629	39 F	258/138	0	17	84	97	340	2.9	5.3	0.6	233	71
10	9222	38 M	168/100	0	10	92		600	7.7	12.3	2 3	233	78
11	8307	36 M	210/130	0	14	133	108	786	7.8	9.5	2.8	178	
12	8279	41 F	224/128	0	11	130	91	396	5.1	10.5	2.1	189	71
13	8277	47 F	226/130	0	14	77	92	475	5.8	9.6	2.4	183	73
14	8559	26 F	178/124	0	10	87	.	461	7.1	8.5	2.8	290	88
15	8267	42 F	230/124	0.5	25	40	68		5 5	8.2	1.3	253	53
16	8486	45 M	250/152	4.4	78	7	38	600				202	63
					Average.........			537	6 6	10 6	2.5	213	80
					Standard deviation...........			±147	±2.2	±3.0	±1.0	±46	±16
					Normal average‡.			566	7.7	11.1	3.2	232	82
					Normal standard deviation‡.....			±166	±3 0	±4.7	±1 6	±62	±17

† All hemoglobin determinations by O_2 capacity. Percentages calculated assuming normal for men to be 20.7 volumes per cent O_2 capacity, for women 19.0 (Peters and Van Slyke, Vol. I, p. 544 (1931)). Plasma proteins were normal in all cases.

‡ From Page, Kirk, Lewis, Thompson, and Van Slyke (1935), Table IV.

Cases of essential hypertension studied. The cases reported in Table I were classified as essential hypertension on the following grounds.

They showed hypertension, which was idiopathic insofar as could be ascertained: in none of the cases had the hypertension been preceded by nephritis or other observed disease of probable significance. In all except the last 2 cases in the table the hemoglobin contents and urea clearances were still within or near the normal ranges. In Cases 15 and 16 shrinkage of the urea clearance had occurred, and decrease of hemoglobin. These were, however, late effects of a condition that had run a course typical of essential hypertension: the cases are therefore placed in this group. In all cases the plasma protein concentration was normal, a significant point in differentiating from chronic nephritis. In all cases except Number 16 urinary protein was absent or slight. The eyeground changes consisted of varying degrees of arteriolar constriction, perivasculitis, tortuosity of the vessels, and arteriovenous compression. Only 1 of the 16 patients was over 50 years of age.

The patients reported were all in fairly good physical condition, were satisfactorily consuming the usual ward diet, and were apparently free from complications such as might be expected to affect the blood lipids (for discussion of such conditions, physiological and pathological, see the chapter on lipoids in Peters and Van Slyke (1931)).

Results with essential hypertension. The data in Table I do not indicate the slightest tendency towards abnormality, in the total plasma lipid content, in the combined or free cholesterol, or in any other of the lipid fractions determined.

DISCUSSION

Our results confirm those of Loewenstein (1928), Weinstein and Weiss (1931), Hoesch (1931), and Bürger and Möbius (1934), and are contrary to those of the authors, cited above, who observed increases of total or free cholesterol, or of plasma lipids, in essential hypertension. Nor do we find any abnormalities in the ratio of free to total cholesterol, or of any individual lipid to the total lipid content, when our results are compared with those obtained by Page, Kirk, Lewis, Thompson, and Van Slyke (1935) from normal men by the same methods used in the present work.

The causes which have led to numerous reports of cholesterolemia

and lipemia in cases of hypertension are uncertain, but the following appear to be possible explanations:

1. The cases reported may in part not have been of the type which we define as essential hypertension. The clinical data given are too slight as a rule to outline the basis of the diagnoses. Loewenstein and Wacker and Fahrig have specified the cases in which they diagnosed the hypertension as essential, but some of the other authors have apparently not attempted a differentiation. In some cases the hypertension may have accompanied hemorrhagic nephritis, with more or less of the nephrotic syndrome, and the accompanying lipemia.

2. Some of the cases of essential hypertension reported may have been complicated by other nutritional, physiological, or pathological conditions conducive to lipemia. Our patients were apparently free from such complications, were receiving and consuming a normal diet, and the blood was drawn during the morning fast.

3. The assumed normal ranges of plasma lipid concentrations used as a basis of comparison may have been too low. With the exception of Kirchgessner (1934), none of the authors quoted above as finding high total or free cholesterol in hypertension has apparently controlled his results by comparison with analyses made by himself with the same methods on strictly normal plasma. Wacker and Fahrig do indeed report control analyses on 19 "Gesunde und Rekonvalescenten," who, however, were not all entirely healthy subjects. The normal ranges of Wacker and Fahrig for total lipids and total cholesterol are lower than those of Page, Kirk, Lewis, Thompson, and Van Slyke (1935). For total cholesterol the previous authors who have been quoted, except Kirchgessner (1934), have apparently assumed 160 or 180 mgm. per 100 cc. of plasma to be the upper limit of the normal range. Kirchgessner states, without giving data, that he finds 300 mgm. the upper limit by the colorimetric method, and 180 by the gravimetric digitonin procedure. The data of Page, Kirk, Lewis, Thompson, and Van Slyke (1935), by digitonin precipitation, indicate 232 mgm. as the mean normal value for total cholesterol, 307 mgm. as the limit exceeded by 1 normal out of 10, and 335 as the limit exceeded by 1 out of 20.

4. The methods of lipid analysis used in the present study are differ-

TABLE II

Malignant Hypertension

Subject			Clinical data						Lipids per 100 cc. plasma						Total N/P atomic ratio	Amino N/P atomic ratio
Series number	Hospital number	Age and sex	Date	Blood pressure	Urine proteins for 24 hours	Blood urea nitrogen per 100 cc.	Urea clearance	Hemoglobin	Total lipid C	Total lipid P	Total lipid N	Lipid amino N	Cholesterol			
													Total	Free		
		years		*mm. Hg*	*grams*	*mgm.*	*per cent*	*per cent**	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*		
1	9050	48 M	February 14, 1934	224/142					750	13.0	49.9	11.5	284	91	8.5	1.96
1	9050	48 M	April 11, 1934	260/170	2.7	21	69	96	608	8 6	12.2	4.8	221	98	3.1	1.23
1†	9050	48 M	April 16, 1934						831	7.9	13.6	4.1	314	123	3.8	1.15
2	8784	31 F	October 1, 1933	220/160	9 3	16	31	115	859	13.0	17.4	6 1	371	97	3.0	1.05

* 100 per cent Hb, 20.7 volumes per cent O_2 capacity for men, 19.0 for women.

† Blood taken after terminal apoplectic stroke.

ent and, we believe, more accurate than those employed in most previous studies of hypertension. The cholesterol determinations, in the papers discussed above, have all been done colorimetrically except those of Bürger and Möbius and 1/3 of those of Kirchgessner. Mühlbock and Kaufmann (1931) found by the colorimetric method of Bloor from 176 to 82 per cent as much serum or blood cholesterol as by the digitonin method, and Kirchgessner found similar discrepancies, attributable to errors in the colorimetric method. For other lipids, apparently only Wacker and Fahrig followed Bloor's modern combustion technique or procedures of comparable accuracy.

That very high plasma cholesterol content in man, when it does occur, may possibly predispose to arteriosclerosis is indicated by observations on diabetic patients. Bloor, Buckner, and Gibbs (1932) found that the group of diabetics with the most advanced sclerosis showed the highest total and esterified plasma cholesterol contents, and that the proportion of cholesterol in esterified form was 10 to 15 per cent above the usual normal value. White (1934) found that diabetics with high plasma cholesterol contents were 15 times as likely to develop arteriosclerosis as diabetics with normal cholesterol.

These findings would support the possibility of hypercholesteremia as a causative factor in the arterial changes of essential hypertension, however, only if, in this disease, high plasma cholesterol could be shown to occur before or during the development of these changes. On the contrary, our results indicate that essential hypertension, if uncomplicated by other disorders tending to produce lipemia, is not accompanied by any disturbance of the plasma lipid picture.

Malignant Sclerosis

Only two cases were available for these studies. The results are presented in Table II. The lipid values fall in or above the upper ranges found in normal subjects.

SUMMARY

In 16 cases of uncomplicated essential hypertension the plasma lipids were determined by the methods of Kirk, Page, and Van Slyke (1934). In no case was the concentration of total lipids or of any of the lipid fractions outside the usual range of normal values, nor were

the means and standard deviations of the group significantly different from those found in a group of normal subjects. Theories of the genesis of arterial changes in essential hypertension based on presumed hypercholesteremia, or on elevation of the cholesterol: phosphatide ratio, are, according to our results, without basis.

Only 2 cases of malignant sclerosis were available. They showed lipid concentrations in or above the upper ranges found in normal subjects.

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SULFHYDRYL AND DISULFIDE GROUPS OF PROTEINS

II. The Relation between Number of SH and S-S Groups and Quantity of Insoluble Protein in Denaturation and in Reversal of Denaturation

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INTRODUCTION

In a previous paper methods were described for estimation of the sulfhydryl (SH) and disulfide (S-S) groups of proteins. It was shown that in a denatured but unhydrolyzed protein the number of groups detectable is the same as that found in the hydrolyzed protein (Mirsky and Anson, 1934–35).[1] With the same methods, applicable to native as well as to denatured proteins, it is found that in some native proteins no SH and S-S groups can be detected, while in others a fraction of the number present when the protein is denatured can be detected. In this paper it is shown that when a protein is partially denatured, that is when part of it is converted into a form insoluble under conditions under which the native protein is soluble, the insoluble fraction has the number of reactive SH and S-S groups characteristic of completely denatured protein, whereas the soluble fraction has the number characteristic of protein which has not been denatured at all. When denaturation is reversed, when insoluble protein is converted into soluble protein, groups which have been detectable are so no longer. In the interfacial coagulation of a protein, that is, when a film of insoluble protein forms at the surface of a protein solution, groups appear, the number detectable being the same as that found in the hydrolyzed protein. When a protein is coagulated by irradiation with ultraviolet

[1] This paper will be referred to as (Paper I).

light, groups are detectable in the insoluble protein, and the number of groups is not increased by subsequently exposing the insoluble protein to a typical denaturing agent, such as acid. Finally, when a protein is converted by urea into a form which has an increased number of S-S groups, that form is insoluble in a medium in which native protein is soluble. These new experimental results may be summarized by saying that, in denaturation, formation of insoluble protein and increase in detectable SH and S-S groups are closely linked phenomena. Denaturation, then, is a definite chemical reaction in which different quantitative methods give the same values for the extent of denaturation; a given protein molecule is either completely native or completely denatured. Another conclusion to be drawn from the experimental results now reported is that denaturation is a reversible reaction as indicated not only by changes in solubility but also by changes in the behavior of S-S groups. These conclusions are in harmony with our previous observations on hemoglobin and on trypsin. In the case of hemoglobin, the same results are obtained whether denaturation and its reversal are followed by changes in solubility, in spectrum, or in digestibility (Anson and Mirsky, 1933–34*b*). In the case of trypsin the same results are obtained whether denaturation and its reversal are followed by changes in solubility or in enzymatic activity (Anson and Mirsky, 1933–34*a*).

Appearance of Insoluble Protein and SH Groups in Denaturation by Heat

To investigate the appearance of groups in denaturation the denaturation of egg albumin by heat has been used. In this process both SH and S-S groups appear, but these observations have been limited to the former. Egg albumin solutions were heated so that varying portions (from 16 per cent to 83 per cent) were coagulated. The soluble and insoluble parts of these protein mixtures were separated and each fraction examined for SH groups. No SH groups could be detected in any of the fractions of soluble protein. The method used (Paper I) was to treat the protein with a cystine solution part of which is reduced to cysteine by protein SH groups if any are present. No cysteine was formed. In each of the insoluble fractions, on the other hand, SH groups were detectable. There were now,

indeed, no SH groups in the protein that were not detectable, for there were no additional groups that appeared on hydrolysis. This was demonstrated by treating the protein SH groups with cystine, hydrolyzing the protein, and then finding practically no cysteine in the hydrolysate. A control experiment showed the easily measurable amount of cysteine found in the hydrolysate of coagulated albumin if the treatment with cystine is omitted. Thus in the partial denaturation of egg albumin the soluble protein fractions contained no detectable SH groups; the insoluble fractions contained no SH groups that are not detectable.

Reappearance of Soluble Protein and Disappearance of S-S Groups on Reversal of Denaturation

To study the behavior of S-S groups during the reversal of denaturation serum albumin was used. It has been shown that it is possible to convert about 60 per cent of a denatured insoluble serum albumin preparation into a soluble form indistinguishable from native serum albumin (Anson and Mirsky, 1930–31). These experiments were repeated, and the number of S-S groups estimated in native albumin, in denatured albumin, and in the mixture of reversed and non-reversed albumin. In the denatured protein the number of groups is equal to the number found when the protein is hydrolyzed. Of these groups about 23 per cent are detectable in the native protein. On reversal some of the groups detectable in the denatured protein disappear, the number disappearing being equivalent to that which would be expected if the reversed albumin has the number of groups characteristic of native albumin. Since native serum albumin contains some detectable S-S groups, it is apparent that presence of SH[2] or S-S groups does not in itself indicate that part of a protein system is in the denatured form. What can be said is that when the number of SH and S-S groups increases or decreases that denaturation or reversal of denaturation has occurred.[3] Estimation of these groups can,

[2] Some proteins, in contrast to egg albumin, while in their native state contain a certain number of detectable SH groups.

[3] It will be shown in the following paper that this statement is true only if, when the numbers of groups are estimated, the various protein preparations are under the same conditions.

accordingly, serve as a valuable indication of the occurrence of denaturation in protein systems in which the usual test for denaturation, loss of solubility, is either inapplicable or inadequate.

Appearance of SH Groups in Interfacial Coagulation

When a protein forms a unimolecular film at an aqueous interface, it loses its solubility in water, for if the film is dispersed the protein does not redissolve. If before the film forms, the protein is readily soluble in water, some change in the protein must have occurred during formation of the film. In this respect the "spreading" of a protein film differs from that of an insoluble fatty acid, for when the latter spreads there is no reason to suppose that a change in its constitution occurs. Investigators of protein films, interested mainly in the size and shape of the protein molecule in a film, have paid little attention to possible changes in constitution of the protein. Since the protein in a film is insoluble, coagulated, it is important to compare its chemical properties with those of proteins rendered insoluble by denaturing agents such as heat and acid. For this purpose egg albumin is a suitable protein since it readily coagulates at an air-water interface. A large quantity of coagulated egg albumin is obtained by shaking an albumin solution. As soon as a film forms, it is dislodged by the agitation, leaving place for a new film to form. To estimate the number of detectable SH groups in the insoluble protein, the method (Paper I) used was to treat it with iodoacetate which reacts with SH groups if any are present. The excess of iodoacetate is removed, the protein is hydrolyzed, and the cysteine content of the hydrolysate is estimated. The cysteine content of protein that has not been treated with iodoacetate is also estimated. The decrease in cysteine content of protein treated with iodoacetate is a measure of the number of its SH groups that reacted with iodoacetate. In native egg albumin no SH groups react with iodoacetate; in insoluble albumin all the SH groups contained in the molecule react with iodoacetate, for in the hydrolysate practically no cysteine is found. When ferricyanide is used to react with SH groups, the same results are obtained. With respect to activity of its SH groups, then, egg albumin coagulated at an interface is the same as egg albumin coagulated by typical denaturing agents.

Appearance of Insoluble Protein and SH Groups in Denaturation by Ultraviolet Light

In egg albumin denatured by ultraviolet light, the nitroprusside test shows that SH groups are present. Our experiments demonstrate that in egg albumin denatured in this way, all the SH groups contained in the molecule are detectable, for no increase in the number of detectable groups occurs on adding an excess of acid. The protein rendered insoluble by irradiation with ultraviolet light is treated with iodoacetate, as the result of which the nitroprusside test fails to show any SH groups, and none become detectable after adding an excess of trichloracetic acid.

Appearance of S-S Groups and of Insoluble Protein in Denaturation by Urea

Denatured protein formed in a number of different ways—by heat, acid, alkali, alcohol, surface action, ultraviolet light—manifests SH and S-S groups. After it had been found that urea denatures proteins (Ramsden, 1902, 1913; Anson and Mirsky, 1929–30), Hopkins (1930) observed that in these denatured proteins too, the groups are present. Since so many different agents cause a loss of solubility which is accompanied by appearance of SH and S-S groups, the loss of solubility and the appearance of groups appear to be closely linked. In the denaturation of serum albumin by urea, however, Hopkins believes that he has caused SH groups to appear without the formation of insoluble protein. We have found that the formation of insoluble protein can be demonstrated if the test is properly carried out.

Hopkins observed that when a concentrated urea solution is added to serum albumin there is a prompt appearance of S-S groups but that on removal of the urea by dialysis no insoluble protein can be detected. From this observation, which we have confirmed, he concluded that appearance of S-S groups and appearance of insoluble protein are not necessarily correlated. It should be noted that Hopkins tested for S-S groups *before* the removal of urea and for insoluble protein *after* the removal of urea. We have observed that if the solution of serum albumin in urea is poured into a large volume of concentrated salt solution (in which native serum albumin is soluble) 95 per cent of the protein precipitates. That is, if the tests for S-S groups and insoluble

protein are both made under the same conditions before the removal of urea, then no evidence can be found for the appearance of S-S groups without the appearance of insoluble protein. The reason Hopkins could detect no insoluble protein *after* dialysis (in our experiments a small part of the protein precipitated during dialysis) is that when urea is removed much of the denatured insoluble protein changes into native soluble albumin just as hemoglobin denatured by salicylate (which acts like urea) changes into native soluble hemoglobin when the salicylate is removed by dialysis (Anson and Mirsky, 1933–34 *b*).

EXPERIMENTAL

I. The Heat Denaturation of Egg Albumin

(*a*) *Partial Coagulation.*—A thick paste of egg albumin crystals in ammonium sulfate was diluted with water to obtain a solution containing 0.05 gm. of albumin per cc. For each experiment 30 cc. of this solution were transferred to a 250 cc. centrifuge flask and diluted with 20 cc. water and 20 cc. of a solution prepared by dissolving 20 gm. Na_2SO_4 in 100 cc. of M/5 pH 4.8 acetate buffer. The sodium sulfate-acetate mixture was used to ensure complete precipitation of denatured albumin. The flask containing the albumin solution was placed in a water bath kept at 95°C. As coagulation proceeded the albumin mixture was gently stirred. Times of heating were adjusted so that the quantities of coagulated protein varied from 16 to 83 per cent of the total protein present. After heating, the albumin mixture was cooled under the tap and centrifuged. The supernatant fluid was filtered to remove the few floating particles usually present.

(*b*) *SH Groups of Uncoagulated Protein.*—To the clear filtrate was added an equal volume of saturated ammonium sulfate to precipitate completely the native albumin remaining in solution, and the suspension was filtered. The precipitated albumin, scraped off the filter paper, and transferred to a 50 cc. centrifuge tube, was now ready for estimation of SH groups. No more than approximately 400 mg. of this protein were used. Estimation of SH groups was by the "direct" method (Paper I) in which the protein is mixed with an excess of a cystine solution part of which is reduced to cysteine if reactive protein SH groups are present. The quantity of cysteine formed is equivalent to the number of protein SH groups present. This method, previously used for denatured proteins, may be used for native proteins if the precaution is taken of not making the cystine solution employed so alkaline as to denature the protein. Sodium hydroxide was accordingly added to the cystine until the solution was definitely blue to thymol blue but colorless to thymolphthalein.

(*c*) *SH Groups of Coagulated Protein.*—The coagulated albumin remaining in the flask after centrifuging was thoroughly washed to free it of any adhering native

protein. To do this the precipitate was mixed with 200 cc. of water and 15 cc. of the sodium sulfate acetate mixture, stirred mechanically for 30 minutes, and then centrifuged. When the supernatant fluid no longer gave a precipitate on addition of 20 cc. of a 50 per cent trichloracetic acid solution, washing was stopped. To test for SH groups a portion of the precipitate containing at the most 700 mg. of albumin was transferred to a 250 cc. centrifuge flask, stirred with 200 cc. of water, and 40 cc. of saturated $(NH_4)_2SO_4$, centrifuged and the supernatant fluid discarded. The albumin was suspended in 200 cc. of a cystine solution like that used for estimating SH groups and was gently stirred for an hour, when 25 cc. of 50 per cent CCl_3COOH were added. The mixture was centrifuged, the supernatant fluid being discarded. The precipitated albumin was washed three times with water and trichloracetic acid, dried, hydrolyzed, and finally the cysteine content of the hydrolysate was estimated. The total quantity of albumin that had been coagulated consisted of the part which was treated with cystine and then dried, and of the part which was not treated with cystine. The former was weighed and the latter was estimated colorimetrically with the phenol reagent.

In addition to testing the SH groups of the part of the albumin which had been coagulated by heat and the part of the albumin which was still soluble, we estimated by the direct cystine reduction method the SH groups of albumin made completely insoluble by heat, and we estimated by the colorimetric method previously described (Paper I) the total cysteine content of the hydrolysate of albumin coagulated by trichloracetic acid.

RESULTS

The cysteine content of hydrolyzed egg albumin was 0.616 per cent. Heat coagulated egg albumin contained 0.56 per cent of SH groups. SH groups are recorded in terms of cysteine, that is as the quantity of cysteine which would have the same sulfur content, the amount being expressed as per cent of the total amount of protein. In experiments on partial heat denaturation the quantity of insoluble protein formed varied from 16 to 83 per cent. In no case could any SH groups be detected in the soluble albumin fraction; that is, none of the cystine with which the soluble albumin was treated was converted into cysteine. The insoluble fractions, treated with cystine and then precipitated, washed, and hydrolyzed, formed with phosphotungstate blue colors so faint that the cysteine contents of the hydrolysates could not be estimated accurately, an indication that practically all of the SH groups contained in the protein had been oxidized by the cystine and hence were present in the insoluble, but unhydrolyzed, protein.

II. Reversal of Denaturation of Serum Albumin

Native, denatured, and reversed horse serum albumin were prepared as described by Anson and Mirsky (1930–31). A precipitate of denatured albumin was prepared in the following manner: 400 mg. of the acid-acetone powder of albumin were dissolved in 20 cc. of water. The solution was immersed in boiling water until its temperature reached 95°C. when to it were added a mixture at 100°C. containing 40 cc. M/5 acetate buffer of pH 4.8 and 2.6 cc. N/5 NaOH. The albumin suspension was cooled rapidly to about 40°C. under the tap, divided into 2 equal parts, and centrifuged. The supernatant fluids, in which only a slight haze appeared on addition of trichloracetic acid, were discarded.

The denaturation of one sample of precipitated albumin was partially reversed as follows: The precipitate was suspended in 10 cc. of water, and a clear solution was obtained by adding 3 cc. N/5 hydrochloric acid. To the solution just enough N/5 NaOH was slowly added to re-dissolve the precipitate of albumin that gradually formed. The solution was now blue to brom-thymol-blue and faintly red to phenol red.

The number of S-S groups of partially reversed and of non-reversed albumin was now estimated. For this purpose the first step was to reduce the protein with thioglycollic acid. After reduction SH groups were detectable in the protein whereas originally none was present, and the number of SH groups formed was a measure of the number of S-S groups present in the protein. SH groups formed were estimated by hydrolyzing the protein and estimating the cysteine content of the hydrolysate. The hydrolysate of serum albumin not treated with thioglycollic acid contains no cysteine. The quantity of cysteine in the hydrolysate treated with thioglycollic acid was therefore equivalent to that portion of the cystine of the protein which was originally present as reducible S-S groups in the unhydrolyzed protein. The partially reversed albumin was reduced under precisely the same conditions. To the precipitate of denatured protein were added 15 cc. of a concentrated sodium sulfate solution kept at 30°C. (this solution, to be referred to again, contained 40 gm. of the anhydrous salt dissolved in 100 cc. of water), and in the solution of reversed protein, warmed to 30°C. were dissolved 6 gm. of anhydrous sodium sulfate. A neutralized thioglycollic mixture was made at 30° by adding to 2 cc. of acid enough 0.4 N KOH (dissolved in concentrated sodium sulfate) to make the solution just red to phenol red and then an additional 5 cc. of alkali, 15 cc. M/1 pH 7.3 K_2HPO_4-KH_2PO_4 buffer, 45 cc. concentrated sodium sulfate, and finally 6 gm. of anhydrous sodium sulfate. One-half of this mixture was added to the partially reversed serum albumin and the other half to the non-reversed albumin. The protein suspensions, contained in 100 cc. stoppered Erlenmeyer flasks, were kept at 30° with frequent agitation for 30 minutes. At the end of this time the proteins were freed of thioglycollic acid by prolonged washing with trichloracetic acid (Paper I). Trichloracetic acid was removed from the precipitates by transferring them to 30 cc. collodion tubes and dialyzing in a rocking dialyzer for 5 hours against 0.005 N HCl. It was

necessary to remove trichloracetic acid because otherwise on subsequent addition of acetone, part of the albumin would dissolve. The proteins were then dehydrated in acid-acetone, dried, hydrolyzed, and the cysteine contents of the hydrolysates were estimated by the method previously described (Paper I).

The S-S groups of native serum albumin were estimated in the same way as were those of denatured and partially reversed protein. During the reduction process native albumin was precipitated by sodium sulfate at 30°.

The quantity of albumin that becomes soluble on reversal was estimated in the manner previously described (Anson and Mirsky, 1930–31).

RESULTS

S-S groups are recorded in terms of cystine, that is as the quantity of cystine that would have the same sulfur content, the amount of cystine being expressed as per cent of the total amount of protein.

1. Cystine content of serum albumin—5.6 per cent.

2. S-S groups of denatured, non-reversed serum albumin—5.46, 5.21, 6.08, 6.03—average 5.7 per cent.

3. S-S groups of partially reversed serum albumin—3.37, 2.81, 2.97—average 3.05 per cent.

4. S-S groups of native serum albumin—1.42 per cent.

5. S-S groups appearing on denaturation, (2) minus (4)—4.28 per cent.

6. S-S groups disappearing on partial reversal, (2) minus (3)—2.65 per cent.

7. Per cent of the S-S groups appearing on denaturation that disappear on partial reversal, 6.5—62 per cent.

8. Per cent of the denatured insoluble protein that becomes soluble on reversal—65 per cent.

III. Surface Coagulation of Egg Albumin

Coagulated albumin was prepared by dissolving an ammonium sulfate precipitate of crystalline egg albumin, which contained about 3 gm. of protein, in 500 cc. of water, and shaking the solution in a 2 liter bottle. The bottle was shaken in a cold room at about 5° for 20 hours. Coagulation was complete, for when a little of the suspension was filtered it was found that no precipitate appeared in the filtrate on addition of trichloracetic acid. The suspension was centrifuged, most of the protein forming a cake at the surface. By rubbing the precipitate with a rubber policeman, its physical state was changed so that when suspended in water and centrifuged nearly all of it settled to the bottom of the flask.

The precipitate was divided into three approximately equal parts, one of which

was washed free of salt with trichloracetic acid, dehydrated, and dried. Each of the other parts of the precipitate was placed in a 250 cc. centrifuge bottle and suspended in 100 cc. M/5 pH 9.6 borate buffer. To one suspension was added 15 cc. M/2 potassium ferricyanide and to the other 25 cc. of M/2 sodium iodoacetate (iodoacetic acid neutralized to phenol red with NaOH). After standing for 1½ hours with occasional agitation both bottles were filled with water and centrifuged. The supernatant fluids, in which no precipitates appeared on addition of trichloracetic acid, were discarded. The albumin that had been treated with iodoacetate was washed free of iodoacetate with trichloracetic acid, dehydrated, and dried. The albumin containing potassium ferricyanide was washed until the yellow color of ferricyanide disappeared. This was done by stirring the precipitate with 250 cc. of water, adding 1 cc. of saturated sodium chloride solution to accelerate sedimentation of the precipitate, centrifuging, and then stirring with water again. After removal of ferricyanide the albumin was washed with trichloracetic acid to remove salt, dehydrated, and dried.

Native egg albumin was treated with iodoacetate and with ferricyanide. In 200 cc. of an M/5 pH 9.6 borate buffer 2 gm. of albumin were dissolved. To half of this solution was added iodoacetate and to the other half was added ferricyanide in the concentrations described above. After standing 1½ hours the extent to which the protein SH groups had reacted was determined. In the preparation containing ferricyanide this was quickly done by testing for ferrocyanide. None was present for when ferric chloride was added no prussian blue appeared.[4] The albumin treated with iodoacetate was diluted to 200 cc. with water and then precipitated by addition of trichloracetic acid. The precipitate was washed with trichloracetic acid, dehydrated, and dried.

RESULTS

The various dried albumin preparations which have been described were hydrolyzed and the cysteine contents of the hydrolysates were estimated. The diminution in cysteine content of those preparations treated with iodoacetate and ferricyanide is a measure of the number of reactive SH groups, which are recorded in terms of cysteine:

1. Cysteine content of egg albumin—0.59 per cent.
2. Cysteine content of egg albumin coagulated by shaking—0.55 per cent.
3. Cysteine content of coagulated egg albumin, after reacting with iodoacetate—too little for accurate estimation.
4. Cysteine content of coagulated egg albumin after reacting with potassium ferricyanide—too little for accurate estimation.

[4] A detailed description of this test will be described in a forthcoming paper.

5. Cysteine content of native egg albumin after reacting with iodoacetate—0.57 per cent.

6. SH groups of coagulated egg albumin, that is, (2) minus (3) or (4)—0.55 per cent.

7. SH groups of native egg albumin as shown by the reaction with iodoacetate, that is, (1) minus (5)—none.

8. SH groups of native egg albumin, as shown by the reaction with ferricyanide—none, for no ferrocyanide was formed.

IV. Coagulation of Egg Albumin by Irradiation with Ultraviolet Light

A 1 per cent albumin solution was prepared by dissolving an ammonium sulfate precipitate of crystalline egg albumin in water. 10 cc. of the solution in a shallow layer were radiated with a mercury vapor lamp until approximately one-half of the albumin was coagulated. During the radiation the temperature of the protein solution was not allowed to exceed 35°. The coagulated protein was freed of soluble protein in the manner described above, in Section I *c*. When tested with nitroprusside and ammonium hydroxide, a portion of the coagulated protein gave an intense reaction for SH groups. The rest of the coagulum was treated with iodoacetate the excess of which was removed by repeated washing with trichloracetic acid. The precipitate was neutralized by suspending it in 50 cc. M/10 pH 7.0 phosphate buffer. After centrifuging, no SH groups could be detected in the protein when tested with nitroprusside and ammonium hydroxide.

V. Denaturation of Serum Albumin by Urea

A preparation of horse serum albumin (Anson and Mirsky, 1930–31) dialyzed free of ammonium sulfate contained 6.5 per cent albumin. In 1 cc. of this solution 1 gm. of urea was dissolved, and the solution was allowed to stand for 4½ hours at room temperature. 20 gm. of anhydrous sodium sulfate were dissolved in 100 cc. of a pH 4.8 M/10 acetate buffer. The urea-albumin solution was added to 40 cc. of the sodium sulfate solution. The precipitate obtained was removed by filtration. To 10 cc. of the filtrate were added 3 cc. of 20 per cent trichloracetic acid, and the suspension was centrifuged. The supernatant fluid, which gave no precipitate on further addition of trichloracetic acid, was discarded. The quantity of albumin in the precipitate was estimated by the phenol reagent colorimetric method, using albumin as a standard. It was 5.5 per cent of the quantity mixed with urea. Urea had rendered insoluble 94.5 per cent of the albumin. When 1 cc. of the original native albumin solution was added directly to 40 cc. of the sodium sulfate solution, no precipitate was obtained.

CONCLUSIONS

1. In native egg albumin no SH groups are detectable, whereas in completely coagulated albumin as many groups are detectable as are

found in the hydrolyzed protein. In egg albumin partially coagulated by heat the soluble fraction contains no detectable groups, and the insoluble fraction contains the number found after hydrolysis.

2. In the reversal of denaturation of serum albumin, when insoluble protein regains its solubility, S-S groups which have been detectable in the denatured protein, disappear.

3. When egg albumin coagulates at an air-water interface, all the SH groups in the molecule become detectable.

4. In egg albumin coagulated by irradiation with ultraviolet light, the same number of SH groups are detectable as in albumin coagulated by a typical denaturing agent.

5. When serum albumin is denatured by urea, there is no evidence that S-S groups appear before the protein loses its solubility.

6. Protein denaturation is a definite chemical reaction: different quantitative methods agree in estimates of the extent of denaturation, and the same changes are observed in the protein when it is denatured by different agents. A protein molecule is either native or denatured. The denaturation of some proteins can be reversed.

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SULFHYDRYL AND DISULFIDE GROUPS OF PROTEINS

III. SULFHYDRYL GROUPS OF NATIVE PROTEINS—HEMOGLOBIN AND THE PROTEINS OF THE CRYSTALLINE LENS

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In a denatured but unhydrolyzed protein the number of SH and S-S groups detectable is equivalent to the quantity of cysteine and cystine found in the hydrolyzed protein (Mirsky and Anson, 1934–35).[1] Detectable protein SH groups react with iodoacetate or with oxidizing agents such as ferricyanide and cystine. Detectable protein S-S groups have the property of being reduced to SH groups by a thiol compound such as thioglycollic acid. The methods of detection of these groups are applicable without any modifications to native as well as denatured proteins. When these methods are applied to native proteins which are under the same conditions as are the denatured proteins, some (egg albumin, for instance) manifest no groups at all, while others (serum albumin) show only a fraction of the number present in the denatured form.

Since the typical protein, egg albumin, has no detectable SH groups, it might be suspected that the S-S groups detected in supposedly native serum albumin (Mirsky and Anson, 1935–36) are present only because the procedure for detecting groups has caused some denaturation. In the present investigation, however, SH groups are demonstrated in native hemoglobin, and in this case there is some assurance that the procedure does not cause denaturation, for denatured hemoglobin can readily be detected spectroscopically (Anson and Mirsky, 1925, 1928–29). The disadvantage of using hemoglobin, that heme interferes with estimation of SH groups, can be avoided.

[1] This paper will be referred to as (Paper I).

A study of the SH groups of native hemoglobin shows that the number of groups detectable is dependent on hydrogen ion concentration, the number increasing as the pH rises. At pH 6.8 no groups are detectable but as the pH is raised (in our experiments as far as 9.5) more and more groups appear. When the pH is brought back to 6.8 they are no longer detectable.

These observations raise the question as to how the SH groups of native hemoglobin differ from those of denatured hemoglobin. The difference between the groups of native and denatured proteins is that the latter can be detected at a pH at which the former cannot be. It is found that at pH 6.8, where no groups are detectable in native hemoglobin, SH groups are detectable in denatured globin; and the number detectable is the maximum available; that is, it is equal to the number of cysteine molecules found in hydrolyzed globin. In comparing native hemoglobin with denatured globin, the differences observed may in part be due to the presence or absence of heme as well as to the state of the protein. The same differences, however, are observed between the native and denatured forms of other proteins, the proteins of the crystalline lens, for instance. In general, then, the effect of denaturation is to extend towards the acid side the pH range in which SH groups are detectable. In every thiol compound (thioglycollic acid, cysteine, and glutathione, for example) activity of the SH group in its reactions with oxidizing agents or with iodoacetate increases with a rise in pH, and in this respect both native and denatured proteins resemble other thiol compounds. But even without change in pH, the SH groups of a protein can be activated by denaturation.

The experiments on hemoglobin and the lens proteins show that activation of SH groups can serve as a satisfactory criterion of denaturation. The test for SH groups activated by denaturation should be carried out at a pH so low that the groups of native protein are inactive and yet high enough for those of denatured protein to be active. Proper conditions for the test vary accordingly from one protein to another, for each protein has its characteristic curve relating pH to activity of groups. The SH groups of the native lens proteins become active at a pH below 6.8, those of hemoglobin above pH 7.0, and those of egg albumin are inactive even at pH 9.6.

SH Groups of Hemoglobin

The method used for estimating the SH groups of hemoglobin is substantially the same as the "indirect" method of Mirsky and Anson (Paper I). Hemoglobin at the desired pH is treated with potassium ferricyanide[2] to oxidize any SH groups present, and the excess ferricyanide is removed by dialysis. Globin and heme are then separated by the acid-acetone procedure (Anson and Mirsky, 1929–30) so that the heme will not interfere with the subsequent analytical procedure for oxidized heme reacts with thiol compounds. The SH groups of the denatured globin prepared in this manner are estimated and compared with the number found in globin prepared from hemoglobin which was not treated with ferricyanide. The difference between these values is equal to the number of SH groups oxidized by ferricyanide, and it is accordingly a measure of the number of SH groups present in hemoglobin under the conditions of the experiment.

The number of SH groups found in native hemoglobin is dependent upon the hydrogen ion concentration. At pH 6.8 almost no groups are detectable; in a pH 7.3 buffer, 28 per cent of the total number of groups contained in the protein appear; in pH 9.0, 44 per cent; and in pH 9.5, 65 per cent are found. The effect of change in pH is reversible. If the pH is brought to 8.75 and then, after an interval to 6.8, no groups are detectable at the latter pH.

The experiments at different hydrogen ion concentrations show that the iron porphyrin part of the hemoglobin molecule can be oxidized independently of its SH groups, for at pH 6.8 ferricyanide oxidizes all of the hemoglobin to methemoglobin without oxidizing any of the SH groups. And, conversely, it is possible to oxidize SH groups of hemoglobin without oxidizing other parts of the molecule. This can be done at pH 9.6 by using cystine, a very mild oxidant, to oxidize SH groups without simultaneous formation of methemoglobin.

When the SH groups of hemoglobin are oxidized at pH 9.5, either by ferricyanide or cystine, no denatured protein is present by spectroscopic test (Anson and Mirsky, 1925, 1928–29). If ferricyanide is

[2] The potential of the ferrocyanide-ferricyanide system is constant in the pH range used in these experiments.

used, methemoglobin is formed, and if cystine is used, the hemoglobin remains unchanged spectroscopically; in neither case is any parahematin or hemochromogen, that is denatured hemoglobin, observed spectroscopically.

SH Groups of the Proteins of the Crystalline Lens

The lens proteins are treated with iodoacetate which destroys active SH groups and the excess iodoacetate is removed simply by precipitating the protein with trichloracetic acid and washing. In this

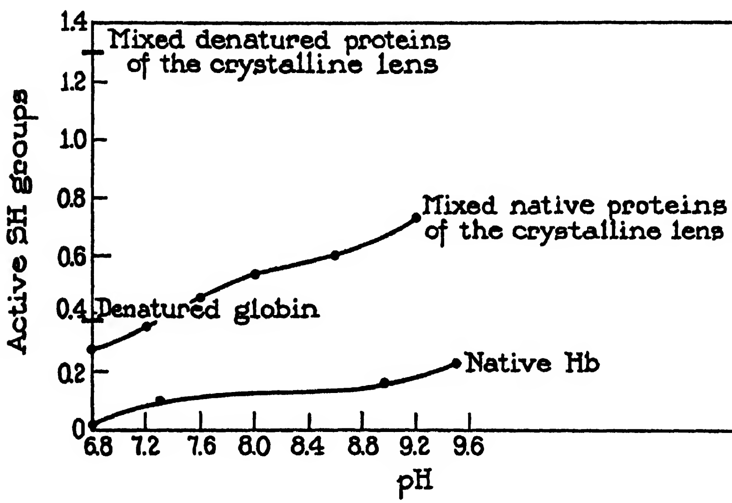

FIG. 1. Relation between pH and number of active SH groups in hemoglobin and in the mixed proteins of the crystalline lens

process the protein is denatured, but in presence of acid the SH groups of denatured proteins are inactive. The number of groups that react with iodoacetate is found by hydrolyzing the protein and estimating its cysteine content. The difference between the cysteine contents of proteins treated with iodoacetate and of those not treated is a measure of the number of SH groups that react with iodoacetate. When these proteins are said to be in the native state, it is meant that in their preparation no agent known to denature proteins is employed. Evidence, such as that available for hemoglobin preparations, that no denatured protein is present, is lacking. And yet a study of the

activity of SH groups indicates that there is little if any, denatured protein in preparations of the lens proteins, for behavior of the groups is similar to that of the SH groups of native hemoglobin. Activity of the SH groups of the native lens proteins is augmented as the pH rises. The effect is reversible; groups activated by a rise in pH lose their activity when the pH drops. When these proteins are denatured, their SH groups become fully active at a pH at which groups of the native proteins are only just beginning to be active.

EXPERIMENTAL

The SH groups of globin were first detected by means of the nitroprusside reaction (Anson and Mirsky, 1930–31). It was then recognized by Schüler (1932) that in the reaction between hemoglobin and ferricyanide these groups, as well as heme, might react with ferricyanide. To estimate the number of SH groups of hemoglobin that might take part in this reaction Schüler titrated globin with ferricyanide. It was assumed that ferricyanide would react only with the SH groups of globin. His experiments have been repeated and his observations confirmed; furthermore, his estimate of the number of SH groups in globin (of the guinea pig) is the same as those we have made (of horse globin) by entirely different methods which appear to be specific for SH groups. When, however, other proteins are treated with ferricyanide we find that there are reducing groups in addition to the SH which react with ferricyanide. These hitherto unrecognized reducing groups of proteins will be described in another paper. Another assumption made by Schüler was that in globin and hemoglobin the same number of SH groups react with ferricyanide. It is shown in this paper that only part of the SH groups of globin are active in hemoglobin. The number active increases as the pH rises. The behavior of the SH groups of hemoglobin should not be neglected in studies on the oxidation-reduction potential of the hemoglobin-methemoglobin system.

It has been claimed by Meldrum (1932) that neither SH nor S-S groups are detectable in globin, and that the observations of Anson and Mirsky can be explained by a failure to distinguish between the color reaction given by nitroprusside with acetone, and that given with SH groups. The evidence for the existence of SH and S-S groups in globin may be briefly summarized:

1. Horse globin prepared by the acid-acetone procedure and then thoroughly washed with trichloracetic acid to remove acetone gives a marked color reaction with nitroprusside and ammonium hydroxide. This color is distinctly different from the color given by acetone. Serum albumin prepared by the acid-acetone procedure and then washed free of acetone does not give a color reaction with nitroprusside and ammonium hydroxide; neither does it contain SH groups, when tested by other methods. This color reaction of horse globin is unmistakable. Only if insufficient care is taken in preparing globin, in which case a deeply pig-

mented protein instead of a colorless one is obtained, are these reactions obscure, as stated by Meldrum.

Globin was treated in the following manner before being tested with nitroprusside; about 100 mg. of protein, with acetone still adhering to it, were mixed with a little water, so as to form first a thick and then a thin paste. About 200 cc. of water were then added and the mixture stirred mechanically for 15 minutes, when 20 cc. of a 50 per cent solution of trichloracetic acid were added. After centrifuging the supernatant fluid was discarded, and the protein was washed again in the same manner. This process was repeated four times. The protein was then washed with sodium sulfate and a pH 7.3 KH_2PO_4-K_2HPO_4 buffer as described below (p. 459). After centrifuging, a little of the protein (now in the form of a very thick paste) was placed on a piece of folded filter paper. The protein was moistened with a few drops of a 5 per cent solution of sodium nitroprusside, the filter paper absorbing the excess fluid. The protein was now moistened with a few drops of dilute ammonium hydroxide.

2. Denatured horse globin reduces cystine to cysteine. This reaction, which is certainly not due to acetone, depends on the presence of SH groups in the protein. The experimental procedure for this reaction is described below.

3. After horse globin has been oxidized with ferricyanide, as described below, it gives no test with nitroprusside for SH groups but it now gives an intense test for S-S groups. Globin treated with ferricyanide does not reduce cystine. To test for S-S groups a few small crystals of potassium cyanide were placed on the protein (on a piece of filter paper, as in the test for SH groups) before adding nitroprusside. No ammonium hydroxide was used (Walker, 1925).

4. When hydrolyzed horse globin reacts with phosphotungstate a blue color is formed indicating the presence of cysteine. Globin oxidized by ferricyanide and then hydrolyzed contains no cysteine. This indicates that before oxidation globin contained a number of SH groups equivalent to the cysteine content of non-oxidized hydrolyzed globin (Paper I).

5. If horse globin is dried in an oven at 110°, in the course of many days its SH groups, as tested for by nitroprusside, gradually disappear; the test for S-S groups does not become negative.

6. Ox globin was prepared by Wu's method, which does not involve the use of acetone, but in which there is ample opportunity for oxidation of SH groups. This globin, when treated with nitroprusside, gives no test for SH groups but does give an intense test for S-S groups.

7. If ox globin, prepared by Wu's method, is treated with acetone which is then washed away, the protein does not now appear to contain SH groups due to traces of acetone which, it may be imagined, were not removed. It gives no color reaction with nitroprusside and ammonium hydroxide, although the protein, due to the treatment with acetone, is so white that even a faint color could be detected. This protein gives an intense test for S-S groups.

Haurowitz (1935) believes that globin contains SH groups, although he was unable to obtain a test with nitroprusside using either ammonium hydroxide or

potassium cyanide. He has suggested that it may be through its thiol groups that globin is attached to heme. This theory, for which Haurowitz advanced no convincing evidence whatsoever, is untenable for several reasons:

1. More than half of the SH groups of globin can be oxidized while the globin is joined to heme in the form of hemoglobin without disrupting the molecule.
2. A molecule of globin of the horse contains only two SH groups, although it can combine with four heme molecules.

Vickery and White (1933) have devised a method for estimating the cystine content of proteins that is entirely different from the method used in our investigations. In their method no distinction is made between cystine and cysteine: quantities of both are lumped together as "cystine" content. Their estimate of the "cystine" content of horse hemoglobin (0.41 per cent) is in good agreement with our value for the *cysteine* content of horse globin (0.42 per cent). This agreement indicates that horse globin contains cysteine but not cystine. Our estimate of cystine content, however, does not agree with that reported by Vickery and White. Since horse globin contains cysteine it is to be expected (Mirsky and Anson, 1930) that an estimation of its cystine content by the Folin-Marenzi (1929) method would be too high, for Folin and Marenzi assume that cysteine and cystine have the same color value with their reagent, whereas cysteine actually gives twice as much color as cystine. (The estimate is 1.14 per cent.) But even after all of the cysteine of globin has been oxidized to cystine, its cystine content, as given by a modification (Paper I) of the Folin-Marenzi method, is 0.63 per cent, distinctly higher than the value reported by Vickery and White.

Cystine may not be the only substance in a hydrolysate of oxidized globin that reacts with the phosphotungstate of Folin and Marenzi in presence of sulfite. That in the hydrolysates of a number of proteins no other substance is present, is shown by the evidence presented in a previous paper (Paper I) and also by the agreement in estimates of cystine content of several proteins by the Folin-Marenzi and the Vickery-White methods. And yet there is evidence that under certain conditions protein hydrolysates do contain interfering substances. We find, using the Folin-Marenzi method, that the cystine content of serum globulin is 2.2 per cent, which is in fair agreement with the value (1.82 per cent) obtained by the Sullivan method.[3] In hydrolyzing the protein 6N H_2SO_4 was used. If more concentrated acid is used, the estimate of cystine content, using the Folin-Marenzi reagent rises to 3.0 per cent if 10N H_2SO_4 is used and to 3.4 per cent if 11.3N H_2SO_4 is used.[4] These results suggest that when globin is hydrolyzed

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[3] Our thanks are due to Dr. Sullivan for the analysis by his method (1926).

[4] These experiments were carried out because of the estimates of cystine content of serum globulin reported by Tuchman and Reiner and by Reiner and Sobotka, using a modification of the Folin-Marenzi method. Their estimates, ranging from 2.34 per cent to 4.70 per cent and averaging 3.64 per cent are distinctly higher and more variable than ours, made with the unmodified Folin-Marenzi method. Their modification was to precipitate the protein with trichlor-

with 6N H_2SO_4 substances interfering with the estimation of cystine by the Folin-Marenzi method may possibly be formed.

It was thought that an estimate of the number of S-S groups in denatured globin by reducing them with thioglycollic acid and estimating the SH groups formed, might serve as a check on the cystine estimation because in some denatured proteins the number of S-S groups is equivalent to the cystine content. Unfortunately estimations of the S-S groups of denatured globin yield results that are both so variable and so high (over 1 per cent) that it appears doubtful whether the method of estimation is applicable to globin. It is possible that thioglycollic acid remains adsorbed to the protein. In the absence of confirmatory evidence it is unlikely that estimation of the cystine content of globin by our method is correct.

Finally, it should be stated that the difficulties encountered in estimating the cystine content of globin do not affect the results reported in this paper, for confidence can be placed in our estimate of the cysteine content of globin. In this case estimation of SH groups serves as a check, since it is found that the number of SH groups in denatured globin is equivalent to the cysteine content of hydrolyzed globin.

Hemoglobin. Reaction between Hemoglobin and Ferricyanide

The reagents used were a 10 per cent solution of horse oxyhemoglobin prepared by Heidelberger's method, M/2 potassium ferricyanide, 3.4 M KH_2PO_4-K_2HPO_4 pH 6.8, M/2 KH_2PO_4-K_2HPO_4 pH 7.3, M/1 K_2HPO_4, M/2 H_3BO_3-NaOH pH 9.0, and M/2 H_3BO_3-NaOH pH 9.6. 5 cc. hemoglobin solution were mixed with 10 cc. buffer and 3 cc. ferricyanide, and the mixture allowed to stand at room temperature for 30 minutes. Under these conditions all the SH groups of denatured globin react with ferricyanide. 8 cc. of the pH 6.8 buffer were then added, and the solution was dialyzed against distilled water in a rocking dialyser for 20 hours to remove the ferricyanide. After adding the various buffer solutions to hemoglobin the pH of the resulting mixtures was measured with the glass electrode, with the following results: after adding the pH 7.30 buffer, the pH of the mixture was 7.30; after adding K_2HPO_4 the pH was 8.75; after adding the pH 9.00 buffer, the pH was 8.94; and after adding the pH 9.6 buffer, the pH was 9.5.

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acetic acid and hydrolyze the precipitated protein with 14N H_2SO_4 instead of drying the protein and hydrolyzing with 6N H_2SO_4. Following their procedure we obtained, in agreement with them, a cystine content of 3.44 per cent. The final concentration of H_2SO_4 in the hydrolysate was about 11.3N. The modification of the Folin-Marenzi method introduced by Tuchman, Reiner, and Sobotka accounts for the high values they obtained, and it probably also accounts for the variability of their results, for it is unlikely that the concentration of H_2SO_4 used by them for hydrolysis was kept constant.

Reaction between Hemoglobin and Cystine

A concentrated cystine solution was prepared by adding to 0.75 gm. cystine N/2 KOH (about 12.5 cc.) until practically all the cystine dissolved, but not enough alkali to make the pH exceed 9.6. The solution was blue to thymol blue but colorless to thymolphthalein. To this were added 5 cc. of a 10 per cent solution of horse carbon monoxide hemoglobin. Carbon monoxide was bubbled through the solution, the flask was then stoppered and allowed to stand in the dark for 1½ hours when 10 cc. of 3.4 M pH 6.8 K_2HPO_4-KH_2PO_4 buffer were added. The precipitated cystine was removed by centrifuging.

Preparation of Globin. The acid-acetone method was used to prepare globin from hemoglobin that had been treated with ferricyanide or cystine. In preparing globin from hemoglobin that had been treated with cystine, it was necessary to add more acid than is usually employed because of the phosphate buffer present. To the hemoglobin solution were added 10 cc. N HCl and to the 600 cc. of acetone used another 10 cc. N HCl were added. Removal of heme made it possible to estimate the SH groups of hemoglobin by the methods used for other proteins. The globin precipitated by acetone was not separated by filtration but by centrifuging. This was done in a 250 cc. centrifuge flask, and the globin was washed free of pigment by further additions of acid-acetone. Most of the acetone was removed by centrifuging, and the rest was removed by washing several times with 5 per cent trichloracetic acid.

Estimation of SH Groups of Untreated and Oxidized Globin.—The SH groups of the various preparations of globin were estimated by the "direct" method (Paper I). Globin was mixed with a cystine solution and the quantity of cysteine formed was equivalent to the number of SH groups of the protein. The number of active SH groups of any given sample of hemoglobin was equal to the difference between the number of SH groups in globin prepared from untreated hemoglobin and the number in globin prepared from hemoglobin that had reacted with ferricyanide or cystine.

Reversal of pH Effect.—To 5 cc. of the hemoglobin solution were added 10 cc. M K_2HPO_4 and after 30 minutes 6 cc. 3.4 M K_2HPO_4-KH_2PO_4 pH 6.8. This globin was treated with ferricyanide and then dialyzed as described above.

The cysteine content (SH groups of hydrolyzed globin) was estimated by the method described by Mirsky and Anson (Paper I).

Reaction of Denatured Globin with Ferricyanide

0.5 gm. of denatured globin in the form of a dry powder prepared by the acid-acetone method (Anson and Mirsky, 1929–30) was dissolved in 50 cc. of water. This was diluted with water to a volume of 200 cc. and to the solution were added 15 cc. of concentrated trichloracetic acid (trichloracetic acid dissolved in an equal weight of water). The mixture was centrifuged, the supernatant fluid discarded, and the precipitate transferred to a 50 cc. centrifuge tube. In this it was well stirred with 40 cc. of a 20 per cent sodium sulfate solution and 10 cc. of a 1.2

M pH 7.3 KH_2PO_4-K_2HPO_4 buffer. The suspension was centrifuged and the precipitate was mixed with 40 cc. sodium sulfate solution and 5 cc. 3.4 M KH_2PO_4-K_2HPO_4 pH 6.8 buffer. After centrifuging, the protein was suspended in a mixture of 25 cc. sodium sulfate solution, 3 cc. pH 6.8 buffer, and 4 cc. M/2 potassium ferricyanide. This tube stood for an hour with occasional agitation and was then centrifuged. The globin was washed with a 10 per cent solution of sodium sulfate by repeated mixing and centrifuging until no ferricyanide could be seen in the washings. The SH groups of this protein were estimated by the direct method, by the quantity of cysteine formed when the protein was mixed with a cystine solution (Paper I). Another sample was hydrolyzed, and the cysteine content of the hydrolysate estimated.

Experiments with the Lens Proteins

Lenses dissected from eyes of oxen were thoroughly mashed in a mortar. During the mashing small amounts of physiological saline were added so that a thin, homogeneous, paste-like mixture of the proteins was prepared. One portion was denatured with trichloracetic acid and then treated with iodoacetate to estimate the SH groups of the denatured proteins (Paper I). Other portions, while in the native state, were mixed with buffers varying in pH from 6.8 to 9.2 and were then treated with iodoacetate. The buffers, all M/2, were phosphate at 6.8, 7.2, 7.6, and 8.0 and borate at pH 8.6 and 9.2. To approximately 7 cc. of a protein mixture (containing about 600 mg. of protein) were added 66 cc. buffer solution and 33 cc. M/10 iodoacetate (iodoacetic acid neutralized with sodium hydroxide). From this point the procedure was the same as that of the "indirect" method for estimating protein SH groups (Paper I). The quantity of cysteine found in the hydrolysate of such a preparation was equivalent to the number of SH groups of the native protein that were *not* active at a given pH. By subtracting the number of inactive groups from the total number present, the number of active groups was obtained.

EXPERIMENTAL RESULTS

SH groups are recorded in terms of cysteine, that is as the quantity of cysteine which would have the same sulfur content, the amount of cysteine being expressed as per cent of the total amount of protein.

Globin and Hemoglobin

1. Cysteine content of hydrolyzed horse globin—0.42 per cent. This is equivalent to 2 molecules of cysteine per molecule of hemoglobin containing 4 iron atoms.

2. SH groups of denatured globin—0.38 per cent.

3. Cysteine content of hydrolyzed globin after denatured globin had been treated with potassium ferricyanide at pH 6.8—Nil.

4. SH groups of denatured globin prepared from hemoglobin treated with potassium ferricyanide at

(*a*) pH 6.8 —0.36 per cent
(*b*) pH 7.3 —0.28 per cent
(*c*) pH 8.96—0.22 per cent
(*d*) pH 9.5 —0.15 per cent

5. SH groups of native hemoglobin at

(*a*) pH 6.8 —(2) minus (4*a*)—0.02 per cent
(*b*) pH 7.3 —(2) minus (4*b*)—0.10 per cent
(*c*) pH 8.96—(2) minus (4*c*)—0.16 per cent
(*d*) pH 9.5 —(2) minus (4*d*)—0.23 per cent

6. SH groups of globin prepared from hemoglobin brought to pH 8.75 for 15 minutes and then treated with potassium ferricyanide at pH 6.8—0.35 per cent.

7. SH groups of globin prepared from carbon monoxide hemoglobin treated with cystine at approximately pH 9.6 (without methemoglobin formation in contrast to the experiments with ferricyanide)—0.14 per cent.

Proteins of the Crystalline Lens

1. Cysteine content of the protein hydrolysate—1.25 per cent.

2. Cysteine content of the protein hydrolysate after denatured protein had been treated with iodoacetate at pH 7.0—Nil.

3. SH groups of denatured protein (1) minus (2)—1.25 per cent.

4. Cysteine content of protein hydrolysate after the native protein had been treated with iodoacetate at

(*a*) pH 6.8—0.97 per cent
(*b*) pH 7.2—0.89 per cent
(*c*) pH 7.6—0.79 per cent
(*d*) pH 8.0—0.71 per cent
(*e*) pH 8.6—0.65 per cent
(*f*) pH 9.2—0.52 per cent

5. Active SH groups of native protein at

(*a*) pH 6.8 (3) minus (4*a*)—	0.28 per cent
(*b*) pH 7.2	0.36 per cent
(*c*) pH 7.6	0.46 per cent
(*d*) pH 8.0	0.54 per cent
(*e*) pH 8.6	0.60 per cent
(*f*) pH 9.2	0.73 per cent

6. Native proteins having been at pH 9.4 for 2 hours in the absence of oxygen treated with iodoacetate at pH 7.2—cysteine content of protein hydrolysate—0.90 per cent.

SUMMARY

Hemoglobin and the proteins of the crystalline lens contain active SH groups while in the native state, the number of active groups increasing as the pH rises. All the SH groups of denatured globin and of the denatured lens proteins are active at a pH so low that practically none of the SH groups of native hemoglobin and of native lens protein are active. The effect of denaturation on the SH groups of a protein is to extend towards the acid side the pH range of their activity.

It is possible to oxidize the iron-porphyrin and the SH groups of hemoglobin independently of each other.

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THE REDUCING GROUPS OF PROTEINS

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In the chemistry of proteins much is known about their acid-base groups but little about other groups. Sulfhydryl and disulfide groups are the only other ones which have been studied with some care, and their importance in investigations of enzymatic activity (Sumner, Lloyd, and Poland, 1933) and protein denaturation (Mirsky and Anson 1935–36*a*) is now recognized. In this paper the existence of other reducing groups in addition to the SH groups is demonstrated and some of their properties are briefly described.

In a previous paper it was shown that ferricyanide can oxidize the SH groups of proteins (Mirsky and Anson, 1934–35). In this paper it will be shown that ferricyanide can also oxidize other groups, that these other groups probably belong to tyrosine and tryptophane, and that the activity of these other groups, as of the SH groups, increases with increase of pH, rise in temperature, and with denaturation of the protein. Whereas the oxidation of SH to S–S by ferricyanide is a definite reaction under suitable conditions—2 SH + 2 ferricyanide = 1 S–S + 2 ferrocyanide—the reaction of the other groups with ferricyanide is not so definite. The greater the ferricyanide concentration and the longer the time of reaction, the more oxidation by ferricyanide takes place. Finally the groups other than the SH groups which are oxidized by ferricyanide are weaker reductants than the SH groups. Conditions can in some cases be found under which they are not oxidized by ferricyanide when under the same conditions protein SH groups are oxidized, and cystine does not oxidize the other groups whereas it can oxidize SH groups.

Knowledge of the new reducing groups is significant in the study of protein denaturation and of the reducing properties of tissues. It can

now be seen that the activation of SH and S–S groups in protein denaturation is part of a more general process. The discovery of the activation of these new reducing groups suggests that still other groups may be activated by denaturation. These reducing groups possess some activity even when a protein is in the native state. The reducing property of the tissue proteins is probably important in maintaining that "reducing environment" which is such a striking characteristic of the interior of the cell.

Method of Detection.—The new reducing groups can be most clearly detected in proteins which contain no SH groups, such as zein and serum globulin. The evidence that reducing groups are present is that when potassium ferricyanide is added to these proteins ferrocyanide is formed. Since ferrocyanide can be estimated as prussian blue, the method is simple, sensitive, and exact. In proteins containing SH groups other reducing groups may be observed if the former are first oxidized. For this purpose a mild oxidant which reacts only with the strongly reducing SH groups is required. Cystine possesses this property (Mirsky and Anson, 1934–35) and by using it to oxidize the SH groups of the muscle proteins, egg albumin and globin, and then treating the proteins with ferricyanide, the other reducing groups of these proteins have been studied.

Factors Affecting the Reducing Groups of Proteins.—The activity of reducing groups depends on whether a protein is in the native or denatured state. In the case of egg albumin this is most clearly observed. Native egg albumin at pH 9.6 does not reduce ferricyanide at all, and since the test for ferrocyanide is highly sensitive even a trace of reducing activity would be detected. Denatured egg albumin, the SH groups of which have been oxidized by cystine readily reduces ferricyanide at pH 9.6. In other proteins the difference between the native and denatured state is not so marked. Native edestin reduces ferricyanide; denatured edestin reduces it twice as rapidly. Muscle proteins which have not been exposed to a denaturing agent reduce ferricyanide almost as readily as do those which have been denatured by trichloracetic acid.

Reducing activity of both native and denatured proteins depends upon the pH; the higher the pH the greater is their activity. The effect of denaturation is to shift the pH range of activity so that a

protein is able to reduce in a more acid medium than it could while in the native state. Reducing activity is also enhanced by a rise in temperature. In edestin a rise in temperature of 10° increases the activity about 45 per cent. Reducing activity varies from one protein to another. Denatured edestin, for instance, is about six times as active as denatured serum globulin, but not quite as active as zein. As already mentioned, the extent of increase in activity on denaturation is a characteristic of each protein.

All these factors—denaturation, hydrogen ion concentration, temperature, nature of the protein—have also been found[1] to influence the activity of protein SH groups. In other ways, now to be described, properties of SH and of other reducing groups differ markedly. Although SH groups are easily oxidized beyond the S–S state, it is possible with ferricyanide (and also with other oxidants) to control conditions so that a clearly defined reaction occurs in which two molecules of ferricyanide react with two SH groups with the formation of two molecules of ferrocyanide and one S–S group. This occurs when denatured globin reacts with ferricyanide at pH 6.8 (Mirsky and Anson, 1935–36*b*). When ferricyanide oxidizes the other reducing groups of a protein the reaction is not clearly defined; the quantity of ferricyanide reduced depends on the concentration used and on the time during which it is in contact with protein. The extent of reduction depends on the concentration of ferricyanide even when the amount of ferricyanide used is 30 to 50 times in excess of the reducing capacity of the protein. A fivefold increase in concentration of ferricyanide may cause a two-and-one-half-fold increase in quantity of ferricyanide reduced. As protein stands in contact with an excess of ferricyanide, the amount of ferrocyanide formed continually increases, although the quantity formed per hour gradually decreases. The limit of the reducing capacity of the protein is not reached even after 24 hours. (In periods as long as this some hydrolysis of protein may occur.)

In the muscle proteins presence of SH groups appears to increase the activity of the other reducing groups. If the SH groups of denatured proteins of muscle are oxidized by cystine and the ability of

[1] In native proteins activity of SH groups increases with a rise in temperature (unpublished experiments).

these proteins to reduce ferricyanide compared with that of proteins still retaining their SH groups, it is found that the latter reduce three times more ferricyanide in the same time. It should be noted that since much of the excess of cystine used to oxidize the SH groups remains adsorbed to the protein, this cystine may in some way affect the other reducing groups.

Comparison of Reducing Capacity and Intensity of SH Groups and of the New Groups

The reducing capacity of these groups in the muscle proteins is more than twice that of their SH groups. On the other hand, the reducing intensity of SH groups is much greater, for they unlike other reducing groups of protein, reduce cystine and phosphotungstate. In denatured globin the difference in reducing intensity of the various groups can be observed in their reactions with the same reagent—ferricyanide. The reducing activity of all these groups increases as the medium is made more alkaline. At pH 6.8 all the SH groups, and only these groups, of denatured globin react with ferricyanide. As the pH rises, other reducing groups become detectable, and at pH 9.6 are quite active.

Identification of the Groups.—Among the known amino acids of proteins those which might possibly be responsible for the reducing properties described above are histidine, tyrosine, and tryptophane. Tyrosine and tryptophane reduce ferricyanide; histidine does not. In the reduction of ferricyanide the general behavior of tyrosine and tryptophane resembles that of proteins. When tyrosine and ferricyanide are mixed, reduction continues for at least 5 hours. The reducing activity increases with a rise of temperature, of pH, or in the concentration of ferricyanide. At 37° 1 molecule of tyrosine can reduce at least 2.6 molecules of ferricyanide. These facts suggest that tyrosine and tryptophane provide the reducing groups of proteins. This conclusion is supported by a study of the reducing properties of zein and gelatin. Although zein contains no tryptophane, it contains much tyrosine, and it actively reduces ferricyanide. A preparation of gelatin containing very little tyrosine reduces only about one-twenty-fifth as much ferricyanide as does an equal weight of edestin under the same conditions.

EXPERIMENTAL

Reagents Used. Proteins.—The egg albumin used had been recrystallized three times. The serum globulin, prepared from horse serum by half-saturation with ammonium sulfate, was a mixture of eu- and pseudoglobulin. The globulin had been freed of albumin by reprecipitation. Zein was prepared by the method of Osborne and Harris. Crystalline edestin was purchased from Hoffmann-La Roche. Mixed proteins of rabbit muscle were prepared by washing finely minced muscle six times with water; the muscle suspended in 20 times its volume of water was stirred for 5 minutes and the water was then decanted. The mixed proteins of frog muscle were prepared in the denatured state (Mirsky and Anson, 1934–35). Globin was prepared by the acid-acetone procedure (Anson and Mirsky).

The potassium ferricyanide used contained no ferrocyanide. Tungstic acid was prepared by adding to 20 cc. of a 10 per cent sodium tungstate solution 960 cc. water and then 20 cc. of $\frac{2}{3}$ N H_2SO_4. A solution of ferric sulfate in gum ghatti was prepared as described by Folin and Malmros.

Procedure.—To several cc. of the protein preparation in a graduated centrifuge tube were added 1 cc. of a buffer solution and several tenths of a cc. M/2 potassium ferricyanide. When the mixture had stood for a definite time, 10 cc. tungstic acid and 0.5 cc. N H_2SO_4 were added and the suspension centrifuged. After the total volume was recorded a measured portion of the supernatant fluid was removed and diluted to 20 cc. with water. To this were added 5 cc. of the Fe_2SO_4 -gum ghatti solution. After 3 minutes, the intensity of the blue color formed was measured in a colorimeter. A blue glass served as a standard which matched prussian blue in the fairly monochromatic light transmitted by a suitable red filter. The standard glass was calibrated by comparison with the prussian blue formed when 0.8 mg. of ferrocyanide was added to the Fe_2SO_4 solution.

The oxidation of proteins by cystine has been described (Mirsky and Anson, 1934–35).

Effect of Denaturation

1. Qualitative Test on Egg Albumin.—To 4 cc. native egg albumin containing 150 mg. protein were added 10 cc. M/2 pH 9.6 borate buffer and 0.3 cc. M/2 potassium ferricyanide. The mixture stood for 30 minutes at 37°C. The same quantity of denatured egg albumin, coagulated by heat, and then oxidized with cystine, was treated in the same manner. On subsequent addition of ferric sulfate, no prussian blue was formed in the solution which had been in contact with native egg albumin; an intense prussian blue was obtained in the other solution.

2. Edestin.—Denatured edestin was prepared by adding to 200 cc. of a 1 per cent solution of edestin, 20 cc. of a 50 per cent solution of trichloracetic acid. After centrifuging and decanting the supernatant fluid, the denatured edestin was washed with water and trichloracetic acid, dehydrated with acid-acetone, and dried (Mirsky and Anson, 1934–35). This powder was dissolved in a half-saturated urea solution, to 1.2 cc. of which (containing 24 mg. edestin) were added 0.3 cc.

saturated solution of urea, 1 cc. M/2 pH 9.6 borate buffer, and 0.25 cc. M/2 potassium ferricyanide. A solution of native edestin was prepared, 2.5 cc. of which contained 24 mg. protein and the equivalent of 1 cc. of the borate buffer. To 2.5 cc. of this solution was added 0.25 cc. M/2 potassium ferricyanide. Both solutions were allowed to stand for 15 minutes at 20°, when to each were added 7 cc. water, 0.5 cc. N H_2SO_4, and 10 cc. tungstic acid. In the solution of denatured edestin, 1.05 mg. ferrocyanide were formed; in the solution of native edestin 0.504 mg. was formed.

3. Proteins of Rabbit Muscle.—Part of the washed muscle was denatured by trichloracetic acid, after which the tissue was washed free of acid (Mirsky and Anson, 1934–35). 5 cc. of this suspension and 5 cc. of tissue which had not been treated with trichloracetic acid were used. To each were added 2 cc. M/2 pH 9.6 borate buffer and 0.7 cc. M/2 potassium ferricyanide. The mixtures were allowed to stand with frequent agitation at room temperature for 1 hour. In each tube were then placed 1 cc. N H_2SO_4 and 20 cc. of tungstic acid. At the end of the experiment the dry weight of tissue which had been treated with trichloracetic acid was found to be 220 mg.; the weight of the other tissue was 228 mg. The former produced 5.56 mg. ferrocyanide; the latter 4.65 mg. It has been found (unpublished experiments) that at pH 9.4 the proteins of muscle are not denatured.

Effects of pH and Temperature:—1.0 gm. edestin was dissolved in 19.5 cc. water plus 0.5 cc. N HCl. To 2 cc. (containing 100 mg. protein) in a 50 cc. centrifuge tube were added 45 cc. water and then 1 cc. of 50 per cent trichloracetic acid. After centrifuging the precipitate was suspended in 45 cc. of a 2 per cent sodium sulfate solution. This suspension was centrifuged and the supernatant fluid was decanted. Such a preparation of edestin was used in each of the experiments to be described. The protein was mixed with 20 cc. of water, 20 cc. of a 20 per cent sodium sulfate solution, 4 cc. M/2 borate buffer, stirred, and then centrifuged. To the precipitate were added 3 cc. water, 1 cc. buffer, and 0.2 cc. M/2 potassium ferricyanide. This mixture stood for 30 minutes, when 15 cc. of tungstic acid and 0.5 cc. N H_2SO_4 were added. The amount of ferrocyanide formed was estimated. At 27° 0.915 mg. ferrocyanide was formed at pH 9.6, 0.65 mg. at pH 9.0, and 0.59 mg. at pH 8.4. At 37° 1.34 mg. were formed at pH 9.6, 0.96 mg. at pH 9.0, and 0.77 mg. at pH 8.4.

Activities of Edestin, Serum Globulin, Zein, and Gelatin Compared

Denatured globulin was prepared by dissolving a little of the ammonium sulfate precipitate in 35 cc. of water, and placing the tube in a water bath, heated to 90°. When coagulation was practically complete, the tube was cooled and centrifuged. The precipitate was washed with a mixture of sodium sulfate and pH 9.6 borate buffer, and subsequently treated with ferricyanide, as in the experiments on edestin. At the end of the experiment the dry weight of the protein was estimated. 100 mg. zein were dissolved in 5 cc. of water plus 0.5 cc. N/2 NaOH. To the solution were added 2 cc. M/2 pH 9.6 borate, 0.25 cc. N HCl (when zein precipitated) and 0.25 cc. M/2 potassium ferricyanide. Reduction

proceeded for 30 minutes at 27°. Under these conditions 306 mg. serum globulin formed 0.49 mg. ferrocyanide, 100 mg. zein 1.34 mg. ferrocyanide, and 100 mg. edestin 0.195 mg. ferrocyanide. To compare gelatin with denatured edestin the latter must be dissolved in urea, as previously described. 120 mg. gelatin were dissolved in 3 cc. of water to which were added 1 cc. M/2 pH 9.6 borate buffer and 0.3 cc. M/2 potassium ferricyanide. In 30 minutes, at room temperature, 0.33 mg. ferrocyanide was formed. Under the same conditions about one-sixth (21 mg.) of the quantity of edestin formed 1.4 mg. of ferrocyanide.

Effects of Time and Concentration of Ferricyanide

In experiments on denatured edestin dissolved in urea, as described above, at 25° 21 mg. edestin when mixed with 0.25 cc. M/2 potassium ferricyanide formed 0.99 mg. ferrocyanide in 15 minutes, and 1.4 mg. in 30 minutes. When only 0.1 cc. of ferricyanide was used, 0.56 mg. ferrocyanide was formed in 30 minutes, and with 0.5 cc. ferricyanide 1.83 mg. ferrocyanide were formed.

100 mg. precipitated denatured edestin were treated with ferricyanide at pH 9.6 as described above. After the mixture had stood at 37° for 24 hours, 40 cc. of water in 2 cc. of a pH 6.8, 3.4 M KH_2PO_4-K_2HPO_4 buffer were added. The suspension was centrifuged. The precipitate was washed in a 2 per cent sodium sulfate solution, by being repeatedly suspended and then centrifuged, until no yellow pigment remained. The edestin was then again mixed with borate and ferricyanide and allowed to remain at 37° for 30 minutes. During this period 0.72 mg. ferrocyanide was formed. Under these conditions, freshly prepared denatured edestin formed 1.34 mg. ferrocyanide.

Effect of Presence of SH Groups on Activity of Other Reducing Groups

Minced frog muscle was thoroughly washed with trichloracetic acid (Mirsky and Anson, 1934–35) to remove soluble reducing substances. The cysteine content of one portion was estimated. Another portion was oxidized with cystine. The reducing activity of this sample was compared with that of another sample which still contained SH groups. Both samples were transferred to 50 cc. centrifuge tubes and there washed several times with water. Each preparation was then suspended in 40 cc. of a 10 per cent sodium sulfate solution plus 5 cc. M/2 pH 9.6 borate buffer. After centrifuging, to each precipitate were added 2 cc. of water, 1 cc. buffer, and 0.5 cc. M/2 potassium ferricyanide. The mixtures were allowed to stand with occasional agitation at 20° for 1 hour. In the tube containing muscle oxidized by cystine, 3.32 mg. ferrocyanide were formed and the dry weight of the protein was 644 mg. This is equivalent to 0.516 mg. ferrocyanide for 100 mg. of protein. In the other tube 5.96 mg. ferrocyanide were formed and the dry weight was 231 mg.—2.58 mg. ferrocyanide for 100 mg. of protein. From this figure must be subtracted 1.00 mg. ferrocyanide which was formed by the SH groups of the proteins (the cysteine content of which is 0.58 per cent). This leaves 1.58 mg. ferrocyanide as having been formed by the non-SH reducing

groups of the proteins containing SH groups, as against 0.516 mg. ferrocyanide by those groups in the proteins with oxidized SH groups.[3]

Reducing Properties of Tyrosine and Tryptophane

100 mg. tyrosine were dissolved in 98 cc. of water plus 2 cc. M/2 pH 9.8 borate buffer and 100 mg. tryptophane were dissolved in 100 cc. of water. In all experiments 0.5 cc. tyrosine or 1 cc. of tryptophane solution were mixed with 1 cc. M/2 borate buffer and (unless otherwise stated) 0.2 cc. M/2 potassium ferricyanide. After a definite interval of time 17 cc. of water, 0.5 cc. N H_2SO_4, and 5 cc. of ferric sulfate-gum ghatti were added. At pH 9.6 and at room temperature tryptophane formed 1.21 mg. ferrocyanide in 30 minutes; at pH 8.4, 0.272 mg. was formed. Under these conditions tyrosine formed 1.02 mg. at pH 9.6 and 0.745 mg. at pH 9.0. Tryptophane at pH 9.6 formed 0.48 mg. ferrocyanide in 2 minutes, 1.21 mg. in 30 minutes, 1.73 mg. in 60 minutes, and 2.68 mg. in 120 minutes. Tyrosine at pH 9.0 formed 0.38 mg. ferrocyanide in 5 minutes, 0.57 mg. in 25 minutes, 0.82 mg. in 70 minutes, 1.01 mg. in 190 minutes, and 1.44 mg. in 3000 minutes. Tryptophane at pH 8.4 in presence of 0.4 cc. M/2 potassium ferricyanide formed 0.53 mg. ferrocyanide in 50 minutes at 26°; at 36° it formed 0.99 mg. On the other hand, at 16° and at 23°, tyrosine formed about the same quantity of ferrocyanide. Tyrosine at 31° and pH 9.6 in presence of 2.0 cc. of M/2 potassium ferricyanide formed 1.25 mg. ferrocyanide whereas in presence of 2 cc. of M/20 potassium ferricyanide it formed 0.24 mg. ferrocyanide.

SUMMARY

1. Intact, unhydrolyzed proteins possess in addition to SH groups other reducing groups which can be oxidized by ferricyanide.

2. The activity of these reducing groups, like that of SH groups, is enhanced by denaturation of the protein and by increase of pH and temperature.

3. These groups differ from SH groups in the manner in which their activity is dependent on concentration of ferricyanide and time of contact with ferricyanide.

4. The activity of these groups is increased if protein SH groups are present.

5. The number and activity of these groups varies from protein to protein.

6. These groups are probably contained in the tyrosine and tryptophane components of proteins.

[3] This experiment should be repeated with muscle proteins carefully freed of lipoids. Adhering lipoids may be responsible for some of the effects observed.

7. The significance of these reducing groups for an understanding of protein denaturation and the reducing properties of tissues is indicated.

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FIELD EXPERIMENTS WITH THE JAPANESE BEETLE AND ITS NEMATODE PARASITE*

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WITH A STATISTICAL ANALYSIS BY J. W. GOWEN

INTRODUCTION

In 1929 a nematode parasite of the Japanese beetle (*Popillia japonica* Newm.) was discovered in one locality in New Jersey (1). The same year Steiner (2) placed the form among the Oxyuridæ, and described it as a new genus and species under the name of *Neoaplectana glaseri*. In 1931 (3), the senior author reported the cultivation of the parasite on an artificial medium, and the following year (4) he published some detailed studies on the subject. Among other matters, it was found that experimental infections of healthy grubs with the second-stage nemas caused a high mortality among beetles in the grub and pupal stages. The parasites infected the host by way of the mouth, developed two or three generations within the body and destroyed the grubs by feeding upon their tissues. The development of *Neoaplectana* continued within the grub cadavers until most of the tissues had been consumed. In dying and newly dead individuals all stages of nematode development were found; in cadavers that had been dead longer, the second-stage or free-living invasive form dominated. These free-living forms vacated the grub remains to seek other victims after everything had been consumed. The entire life cycle of *Neoaplectana*, corresponding to the life history within the host, was successfully cultivated upon a special artificial medium (3, 4). A generation developed every 4 or 5 days and cultivation apparently did not alter the pathogenicity of the nemas for Japanese beetles. Preliminary field experiments, on a small scale (4), indicated that the parasite could be established in a region where it did not occur naturally and, when so established, produced a high mortality.

The field experiments initiated in 1931 on a small scale were con-

* Conducted cooperatively by The Rockefeller Institute for Medical Research, Princeton, N. J., and the New Jersey State Department of Agriculture.

tinued. It seemed desirable to determine whether or not the introduced parasites had permanently established themselves; in other words, whether they could remain dormant through successive winters and become parasitically active again during the warm months.

Large field experiments were also planned and executed with two chief questions in mind. First, how can the nematodes be best introduced and established on a large scale in a region so that they will become an important control factor? In the solution of this problem the number of the host population in an area must be considered, as well as the actual method for introducing the parasites. Second, with all the variable factors which are encountered in the field, is it possible to obtain quantitative results which will enable an appraisal of the extent of parasitism and the consequent reduction in host population? Quantitative data, on work with insect parasites generally, are much needed at the present time. No criticism can be made of those few striking cases where a complete or nearly complete extermination of a pest has been accomplished by means of introduced parasites. In many instances, however, extermination of a noxious insect by introduced foreign parasites has not been effected. Some of these parasites established themselves and a measure of control is claimed for them, but the chief question cannot be answered from the available data. The problem of how much host reduction the parasites accomplish—*i.e.*, how effective the parasites really are, remains unsolved. In fairness to certain workers with insect parasites, it must be stated that the Japanese beetle during the grub stage lends itself very well to investigations of a quantitative nature. The larvæ live in the soil and consequently, by making a large number of standard diggings and counting the larvæ so obtained, a fairly reliable index of the population from year to year can be obtained. The effect of a parasite on such a population should yield fairly reliable results. One section of the present paper deals with quantitative aspects obtained during the course of some field work with the nematode parasite of Japanese beetle grubs.

Further Observations on Two Small Plots Inoculated with Nematodes in 1931

In the spring of 1931, as described in a previous publication (4), two localities in southern New Jersey were chosen on two separate farms

about two miles apart. On each farm an experimental and a control plot, separated from each other by 150 yards, were selected. Each plot comprised 6 square feet and originally contained between four and five hundred grubs. Boards were driven one foot into the ground to enclose the grubs and to prevent their lateral migration out of the plots. During the summer the insects remain in and near the root system of plants and do not migrate vertically more than 3 or 4 inches.

On May 15, 1931, the soil within the four plots was carefully sifted to a depth of over 6 inches and all of the grubs examined and counted. They were all found to be healthy and in the second and third instars.

TABLE I

Adult Beetle Emergence over a Period of Four Years in Experimental Plots

Year	No. of grubs in each plot	Adult Emergence				Remarks
		Experiment A		Experiment B		
		Control plot	Infected plot	Control plot	Infected plot	
1931	600 in A plots 450 in B "	50	1	175	30	Each of 6 adults collected from Infected Plot B harbored between 5–8 nemas
1932	200	128	8	158	0	Each of 4 adults collected from Infected Plot A harbored a large number of nemas
1933	200	158	0	143	0	
1934	300	146	0	180	0	

On one farm, the grubs were equalized to 600 in each plot. On the other farm, the grubs were equalized to 450 in each plot. Since the grass was entirely uprooted and injured during this procedure, rye for food was heavily sown in the four plots.

On May 18, the soil in one plot, A, on the first farm, was treated with a culture of *Neoaplectana* and on May 22, the procedure was repeated on Plot B, on the second farm. The control plots on each farm remained untreated throughout the season. The method for preparing and applying the nematode cultures was described in the previous publication.

During the entire season of 1931, 47 parasitized grubs were found in inoculated Plot A and 64 in inoculated Plot B. No cases of parasit-

ism were found in either of the control plots. Table I gives the number of adult beetles that emerged from each plot. Counts showed that a large number of grubs were being lost from some cause other than parasitism. It was discovered that birds were a factor, so screened cages were placed over each plot. However, in spite of the enormous losses, several points were evident. The parasite was established in the field in a region where it did not naturally occur and produced a high mortality, although the percentage of mortality from nematodes cannot be computed because of unfortunate losses from other causes. The number of deaths by agents other than nematodes was approximately the same in each of the four plots.

During 1931 after the emergence of the adults, soil was frequently sedimented in a "Baermann isolation apparatus" to see if the second-stage nemas were still active. Each test revealed many of the parasites which were always cultured to the adult stage for accurate identification. *Neoaplectana* was not recovered from similar samples of soil taken from the control plots nor from samples of soil from eleven other localities in the vicinity. Samples immediately outside of the infected areas were also frequently tested during 1931 for the presence of the parasite with negative results. Therefore no evidence on the migration of *Neoaplectana* from its place of introduction was obtained at this time. However, it will be recalled that, for the purpose of the experiments, the grubs were prevented from migrating laterally. This restraint probably also assisted in keeping the nematodes within the circumscribed area.

The small plots were studied from 1931 through 1934 (Table I). Each fall after the adult emergence a certain number of grubs were always added to the soil in each plot. In the spring two grub equalizations were made; one in April, the other during the latter part of May. In the plots inoculated with nematodes the first lots of grubs were always so reduced numerically by the parasites within 3 or 4 weeks that a second grub introduction was made. After the pupæ are fully formed, at the middle of June or later, grubs that have escaped infection or are possibly immune reach maturity.

The figures in Table I, second column, represent the numbers of grubs added each year during the last equalization. In infected Plot A no adults have emerged during the past 2 years, and in infected

Plot B none have appeared since 1931. The plots were examined at intervals for parasitized grubs. During 1932 infected Plots A and B yielded 133 and 93 cases, respectively. During 1933 Plot A yielded 48 cases; Plot B was only disturbed once that year when 5 cases were collected. During 1934, 15 cases were found in Plot A and 46 in Plot B. No parasitized cases were ever found in the two controls. The data given do not present a complete record of all of the parasitized material. The observations were necessarily intermittent and during the intervals many cases undoubtedly disintegrated beyond recognition. Only diseased individuals and cadavers that revealed large numbers of the specific parasite were recorded.

The table giving the adult emergence shows losses in the control plots. Those for 1931 may be largely accounted for by birds. This cannot be true for the losses sustained during the succeeding 3 years because the plots were screened. Some trouble was encountered during 1932 and 1933 with moles and this was corrected by transferring the soil two feet in depth from each plot to wooden frames with bottoms of copper screen. The plots were now protected from birds, moles, and rodents. Nevertheless, during 1934 the two control plots showed decided losses which may possibly have been due to bacterial and other diseases.

As recorded in the table, during 1931 and 1932 all together 10 adults emerged that harbored second-stage *Neoaplectana* within their intestinal tracts. This observation presents the possibility that the adult beetles, which are vigorous fliers, may assist in the natural dispersion of the nemas.

After emergence in August and September of 1932, 1933, and 1934, samples of soil were sedimented from each of the four plots and in the soil from the inoculated ones, active second-stage *Neoaplectana* larvæ were found in abundance and cultured up to the adult stage. The same tests were repeated with the same result in the early spring before the warm weather had caused the nematodes to become parasitically active among the grubs.

From the evidence presented, therefore, there can be no doubt that *Neoaplectana* became definitely established in the inoculated areas, and caused a high mortality among the Japanese beetle grubs.

Experiments to Test the Possibility of Introducing the Nematodes by Spraying

During the early autumn of 1931, the authors selected some lawn grass which showed considerable damage by Japanese beetle grubs and staked two 15 foot square plots; one was used as a control and the other as an infection experiment. Each area was separated from the other by 15 feet. The grub population averaged $\pm$ 22 per square foot in the control and $\pm$ 29 per square foot in the other plot.*

On the day the nematodes were introduced each plot was first sprinkled with 100 gallons of water. This was considered necessary because the ground, after a prolonged drought was exceedingly hard and dry, and it was thought that the parasites might experience difficulty in penetrating the surface. Four large pie plate cultures** with a heavy growth of second-stage nemas on the agar yeast medium, previously described (3, 4), were washed off and suspended in 8 gallons of water and with sprinkling cans rapidly distributed over the experimental plot. Subsequently, although many 1 square foot diggings were made, no cases of parasitism were found. Six weeks after the treatment 4 samples each, consisting of 1 lb. of soil taken at four different points in the infected plot at levels from 1 to 6 inches deep and sedimented, did not yield any parasites. Two months later this procedure was repeated with negative results. Ordinary soil nemas were found in abundance, however.

During the early spring of 1932 the grubs in each plot averaged $\pm$ 22 per square foot, showing that no reduction in the population

* The values of 1 square foot diggings were obtained by marking off a square foot on the surface of the ground with the handle of a grubbing hoe notched at the proper length from the end. The turf root system and the soil to a depth of 6 inches were then carefully sifted and examined for grubs. During the warm season grubs remain in and near the root systems of their food plants.

** The pie plates each contained about 60 cc. of dextrose veal infusion agar. The surface was first inoculated with a pure culture of yeast and 24 hrs. later inoculated with second-stage nemas from a Petri plate culture, after washing and sedimenting three times in water. The pie plate cultures were then incubated at room temperature (20–25°C.) for from 2 to 3 weeks. At the end of incubation the nemas had multiplied plentifully, the yeast cells had been consumed, and nearly all of the worms were in the second-stage which is the only stage that survives in the soil. (See literature citations 3 and 4.)

had occurred and no cases of parasitism were found. During the middle of June the average grub count for the control plot was ± 20 and for the inoculated plot ± 15. This slight reduction is within the bounds of experimental error and signifies nothing in so far as the treatment is concerned, because no cases of parasitism were uncovered.

The reason for the failure of this attempt at introduction is difficult to interpret. The method used was similar to the one used on the small plots which were so successful. However, the drought and condition of the soil may have prevented the nemas from penetrating. If they penetrated through cracks and crevices, they may have found conditions too dry in spite of the preliminary moistening, which probably had little effect on the soil below the surface. The grub population, and the dosage of nematodes used may also have been important factors.

In the autumn of 1931, a section of a timothy and clover pasture, showing considerable grub damage, was enclosed by a wire fence to keep out cattle. This area measured 450 feet by 50 feet and yielded an average grub count of ± 31 per square foot. The enclosed area was divided into three plots each measuring 130 feet by 30 feet. These were so spaced that each was surrounded by a so-called neutral area. A space of 20 feet existed between the plots and 10 feet between them and the fence surrounding the entire enclosure. The timothy and clover were first cut to prevent the nemas from lodging on the vegetation where they would have been rapidly desiccated. The three plots were then well watered from a clean power sprayer. One of the three remained untreated as a control. The second was treated by evenly distributing with sprinkling cans, 6 heavy pie plate cultures in doses of 1 pie plate culture to 2 gallons of water. Although most of the nemas were carefully washed off the surface of the agar, some of them stuck to the medium, so the agar was cut into small pieces and also broadcast over the surface of the ground. The ground was again gently sprinkled with the power sprayer to wash off those nemas that had lodged on the vegetation.

The third plot was treated directly with the power sprayer. This sprayer, of 300 gallons capacity, had not been used for insecticide work for an entire year. The tank, pump, pipes, hose, etc., had been thoroughly washed and treated to eliminate all traces of poison. For

the inoculation the surface growth of 7 heavy pie plate cultures was washed into the tank containing 250 gallons of water. The agitator was rotated slowly to keep the worms in suspension and very little pressure was used. Samples of water taken from the end of the spray nozzle showed that the parasites issued alive at the rate of about 5 to 10 per 10 cc. of water.

Subsequently, the three plots were frequently visited and from between 10 and 20 one square-foot diggings made on each plot at every examination. No parasitized cases were found until the spring of 1932 when three typical cases were discovered in the plot treated with the sprinkling cans.

From the autumn of 1931 to the spring of 1934, the grub count dropped from ± 31 per square foot to about 12 per square foot without nematode parasitism an apparent factor. However, the grub counts in the control plot during two years dropped far below the counts in either of the infected areas until the spring of 1934 when the mean counts for all three of the plots were approximately equal; 15 for the control, 13 for the hand treated, and 12 for the machine treated sections. A representative number of diggings in the neutral areas yielded approximately the same grub counts and no cases of parasitism.

The above experiment may be summarized by stating that three cases of parasitism were found in the plot treated with the sprinkling cans about 8 months after the introduction of the nematodes. Nothing was found subsequently. The parasite apparently did not become well established and this fact may be correlated with the rapid drop in grub counts due to other factors. Where grubs are numerically low, the chances for the spread of an infection become less. Other factors, as mentioned previously, such as the method of introduction, the dosage or the character of the soil, etc., might also be important. These factors must be determined, and this can only be accomplished through experimentation and observation over a long period of time. Since the parasite became established, although poorly, further studies of this locality are indicated. For instance, the host population may rise appreciably again and if this should occur, as seems likely, the parasite story might assume a different aspect.

In April, 1932, the experiment just outlined was repeated in another

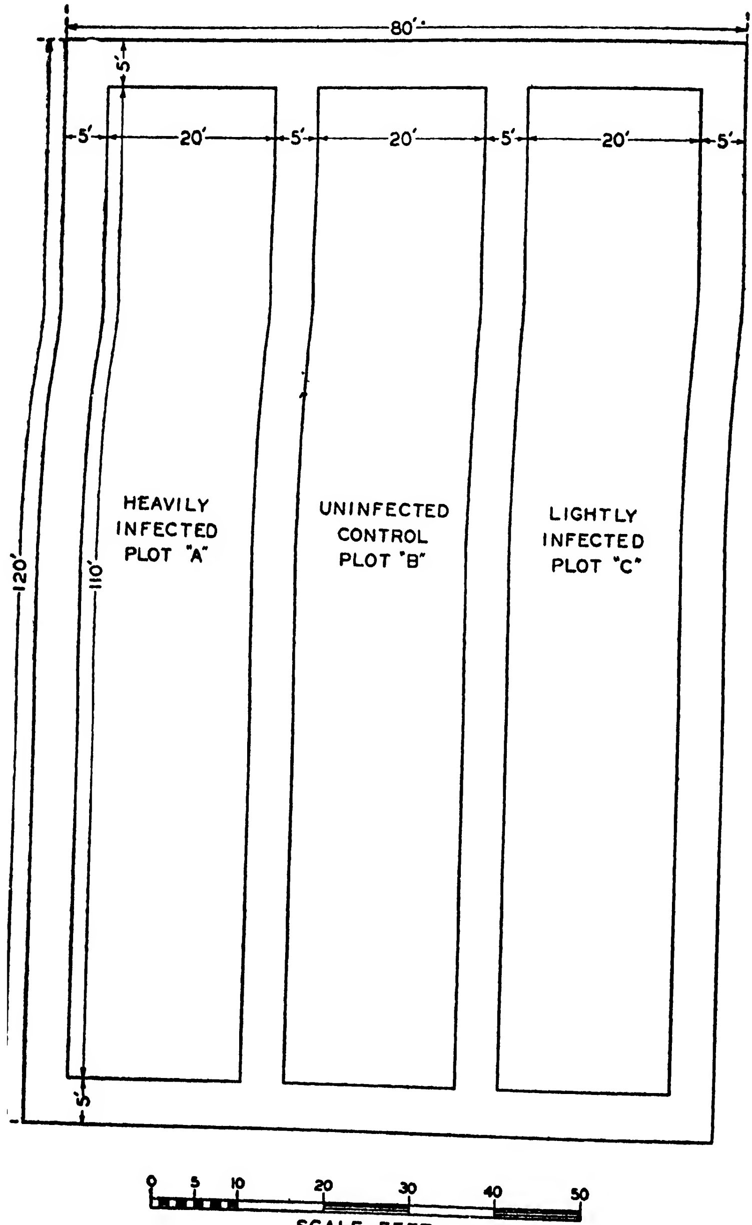
locality. The field used was covered with meadow grass and harbored a grub population of approximately 41 to the square foot. The three plots were handled as above with the exception that the nematode treatment followed a steady, 24-hour rain. From May, 1932, to November of the same year, the grub population gradually dropped to a mean of 3 per square foot. By September, 1933, a rise of 20 to the square foot occurred and in October one parasitized case was found in the hand treated plot. Although 20 diggings were made in each plot at every visit and samples of soil from them were frequently sedimented, the nematodes were not again recovered. One and a half years intervened from the time of introduction until the single case recorded was found, but this poor result may have been due to the almost complete disappearance of the host due to unknown causes. Obviously, it is important to continue the study of this locality, especially as a gradual rise in population again seems to be in progress. It seemed to be impossible to obtain a population that would remain stable for a period of years.

Experiments on the Subsurface Introduction of the Nematodes

To obtain a reasonably heavy and stable population for at least a few years, the next experiment was conducted in a region invaded by the Japanese beetle during the previous year. It has been claimed that a new, heavy infestation lasts for a few years, then declines and may or may not rise slightly again. Evidence as yet does not exist that the infestation goes through a series of high peaks and low points as is the case with some other insects such as the tent-caterpillar. According to the Government entomologists engaged on the Japanese beetle project, the rise and fall of the pests seems probably to be correlated with the extent of rainfall during July and August when the soil population consists of eggs and first-instar larvæ. A deficient rainfall apparently causes a high mortality during the early stages. It is quite possible that, if several favorable years occurred in sequence, a declining population might again rise heavily. Since the main infestation in New Jersey seems to be moving slowly southward, a freshly invaded section was chosen on its southern fringe.

During the spring of 1933 a site was located on a six acre field of pastureland and an area 120 feet by 80 feet was surrounded by a

DIAGRAM OF EXPERIMENTAL PLOTS



barbed wire fence as a protection from cattle and pigs. The grass within the fenced area was mowed and three plots were staked off, in such wise that each measured 110 feet in length by 20 feet in breadth. (See diagram.) Each plot was separated from the other and from the fence by 5 feet. Plot A (diagram) was heavily inoculated with nematodes, Plot B served as an uninoculated control, and Plot C was lightly inoculated. The 5 feet strips that surrounded the plots were designated neutral areas. Tables II, III, IV, and V give, among other data, the average number of grubs per square foot for each plot

TABLE II

Experiment Showing Establishment of Infection at Points of Introduction and Spread away from These Points in Plot A

Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized	Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Prior to parasite introduction						Prior to parasite introduction					
4/20/33	10	953	95.3	0	0	4/20/33	10	953	95.3	0	0
After parasite introduction at stakes						After parasite introduction between stakes					
6/12/33	17	214	12 6	6	2.80	6/12/33	7	121	17.2	0	0
9/28/33	32	1104	34.5	8	0.72	9/28/33	20	741	37.0	12	1.62
5/14/34	75	2496	33.2	64	2.56	5/14/34	75	2032	27.0	43	2.11
6/ 9/34	75	856	11.4	39	4.55	6/ 9/34	75	909	12.0	28	3.08
9/26/34	75	347	4.5	32	9.22	9/27/34	75	490	6.5	25	5.10
10/24/34	75	156	2.08	16	10.20	10/24/34	75	321	4.2	33	10.28

prior to the nematode introduction, and it will be seen that the grub infestation was exceedingly heavy.

Pie plate cultures were prepared on May 9, 1933, from cultures that had been transferred on the artificial medium 7 times at intervals of from 10 days to 2 weeks. By May 23, the cultures were heavy and the nemas were practically all in the second-stage. These cultures were taken into the field and introduced on May 23 and May 29. Seventy-five holes about 3–4 inches deep and spaced approximately 5 feet apart were made on Plot A. One-half of each pie plate culture,

TABLE III

Experiment Showing Establishment of Infection at Points of Introduction and Spread away from These Points in Plot C

Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Prior to parasite introduction					
4/20/33	10	875	87.5	0	0
After parasite introduction at stakes					
6/12/33	10	117	11.7	6	5.13
9/28/33	22	580	26.3	11	1.89
5/14/34	22	460	20.9	13	2.82
6/ 9/34	22	141	6.4	12	8.51
9/27/34	22	129	5.8	3	2.32
10/24/34	22	50	2.2	0	0

Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Prior to parasite introduction					
4/20/33	10	875	87.5	0	0
After parasite introduction between stakes					
6/12/33	10	202	20.2	0	0
10/12/33	15	397	26.4	8	2.01
5/14/34	22	473	21.5	49	10.35
6/ 9/34	22	196	8.9	7	3.57
9/27/34	22	121	5.5	2	1.65
10/24/34	22	107	4.8	1	0.93

TABLE IV

Data from Each Infected Plot Combined

Date of diggings Plot A	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Prior to parasite introduction					
4/20/33	10	953	95.3	0	0
After parasite introduction					
6/12/33	24	335	13.9	6	1.79
9/28/33	52	1845	35.7	20	1.08
5/14/34	150	4528	30.2	107	2.36
6/ 9/34	150	1765	11.8	67	3.79
9/26/34 9/27/34	150	837	5.6	57	6.81
10/24/34	150	477	3.2	49	10.27

Date of diggings Plot C	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Prior to parasite introduction					
4/20/33	10	875	87.5	0	0
After parasite introduction					
6/12/33	20	319	16.0	6	1.88
9/28/33 10/12/33	37	977	26.4	19	1.94
5/14/34	44	933	21.2	62	6.65
6/ 9/34	44	337	7.7	19	5.64
9/27/34	44	250	5.7	5	2.00
10/24/34	44	157	3.6	1	0.64

together with the agar, was placed in each of the 75 holes. All grubs were carefully replaced, the hole watered with a sprinkling can, and the soil and sod replaced. Each hole so treated was marked with a stake and the surface of the ground at each site was again watered. Plot C was similarly treated except that on May 29, 22 holes were made spaced at 18 to 20 feet from one another. Thus only 11 pie plate cultures were consumed, one-half a culture to each site.

Following the introduction, intermittent diggings were made in the three plots and later also in the neutral areas, and in the 6-acre field

TABLE V

Experimentally Uninfected Plot B Which Later Became Naturally Infected

Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Prior to parasite introduction into A and C					
4/20/33	10	787	78.7	0	0
After parasite introduction into A and C					
6/12/33	10	202	20.2	0	0
9/28/33	10	349	34.9	0	0
5/14/34	20	394	19.7	1	0.25
6/ 9/34	20	252	12.6	2	0.79
9/26/34	20	58	2.9	1	1.72
10/24/34	20	39	1.9	0	0
Six acre field outside experimental enclosure					
9/26/34	110	1025	9.3	0	0
10/24/34	100	719	7.19	2	0.28

in which the experimental area was situated. Within the two experimental plots, diggings were made at the stakes (the points of introduction) and halfway between stakes, to obtain an idea of the rate of the migration of the parasites. Diggings in the neutral areas, the control plot and in the field outside all contributed to this rate of migration and to the final estimate of the value of the parasite. At each examination one square foot of earth was dug, the sod was shaken, the soil carefully sifted, and the number of grubs and their instars recorded. A separate record was kept for each digging. A heat

sterilized tin can was reserved for each hole and all diseased, dead, or otherwise abnormal grubs were taken to the laboratory within 12 to 24 hours for microscopical examinations and cultural tests.

The field examinations for grub counts and parasitized material were necessarily widely spaced in time, even during the warm months. This was necessary because of the labor and the cost involved, especially, since the experiments were located at a distance of about 85 miles from the laboratory. In order not to create abnormal conditions, it was also thought best not to disturb the population too frequently.

The tables show the date, month, and year when the examinations were made.

TABLE VI

Neutral Areas Which Became Naturally Infected

Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized	Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Between A and B						Between B and C					
5/24/34	10	218	21.8	0	0	5/24/34	10	217	21.7	2	0.92
6/ 6/34	10	152	15.2	6	3.94	6/ 6/34	10	106	10.6	0	0
9/26/34	10	69	6.9	2	2.89	9/26/34	10	74	7.4	1	1.35
10/24/34	10	46	4.6	2	4.34	10/24/34	10	43	4.3	1	2.32

Table II shows that parasitized material was found in Plot A at the stakes on the first examination made after the introduction of the nematodes. About 3 per cent of the total number of grubs recovered had been killed by *Neoaplectana*. No cases were recovered at that time in the diggings made between the stakes. Subsequently, cases were found at the stakes and between the stakes, at each visit, and the mortality due to nematodes reached 10 per cent of the total grubs recovered in October 1934. These results on this plot show that the parasites established themselves and migrated from the original places where they were introduced. Table III shows similar results obtained on Plot C although the high point of parasitism was reached somewhat earlier. The two sections of Table IV combine

the results from Plots A and C respectively. Table V represents control Plot B and shows that the nemas migrated into this area in May 1934, so from that time on this plot could no longer be considered a control, in the strictest sense of the word. Indeed the same table (at the bottom) gives two sets of diggings in the 6-acre field

TABLE VII

Neutral Areas Which Became Naturally Infected

Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized	Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Along north side of A						Along south side of C					
5/24/34	10	247	24.7	6	2.43	5/24/34	10	124	12.4	3	2.42
6/ 6/34	10	118	11.8	0	0	6/ 6/34	10	95	9.5	0	0
9/26/34	10	109	10.9	8	7.34	9/26/34	10	38	3.8	1	2.63
10/24/34	10	51	5.1	8	15.68	10/24/34	10	28	2.8	0	0

TABLE VIII

Neutral Areas Which Became Naturally Infected

Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized	Date of diggings	No. of diggings	Total No. of grubs	Average per sq. ft.	No. of parasitized cases	Per cent of total parasitized
Along east end of three plots						Along west end of three plots					
5/24/34	10	201	20.1	3	1.49	5/24/34	10	158	15.8	3	1.90
6/ 6/34	10	85	8.5	0	0	6/ 6/34	10	99	9.9	0	0
9/26/34	10	5	0.5	0	0	9/26/34	10	45	4.5	1	2.22
10/24/34	10	15	1.5	0	0	10/24/34	10	23	2.3	0	0

outside the experimental territory and in October 1934, two cases of parasitism were found approximately 20 yards from the fenced locality.

Table VI, VII, and VIII give the results obtained from all of the so-called neutral areas and show again that the nematodes became widespread over the entire enclosed space. Along the north side of A the number of parasitized cases found in October, 1934, equalled over 15 per cent of the total number of grubs recovered on that side.

The number of parasitized cases most frequently found per individual digging equalled 1 and 2 although 3, 4, and 5 were commonly collected and in 1 hole 11 cases were recovered. In general, the number of parasitized individuals found was probably no absolute index of the mortality due to the nemas. However, the percentages parasitized, of the total number of grubs counted, is a more accurate estimate than would be possible by any other method. The data obtained during April, May, and June only cover the larval time up to pupation. It is injurious to the grubs to disturb them when in the process of pupation and a record of a high mortality at this time would be misleading. Data on the extent of the adult emergence during July and early August, as were obtained from the small plots previously discussed, would have also been valuable, but it was impracticable to screen this large area. Autumn examinations were discontinued as soon as cold weather inhibited the grub and nematode activity.

Tables II to V show a general drop in the grub population from April 1933 to October 1934. Some of this downward tendency of the population may be ascribed to the parasites, as will be seen later, but it would be a misrepresentation of facts to ascribe all of it to this cause, because the same tendency was observed in the control plot and in the 6-acre area outside of the experimental enclosure before their invasion by the parasites. Frequently, during the morning hours, the surface of the ground was found riddled with bird holes and starlings, grackles, robins and others were seen feeding upon grubs. Birds, therefore, must have been a factor in this population drop. A similar claim might be made for deficient rain at certain times during the early stages of the insect. Bacterial and other diseases of the grubs were probably also factors.

Notwithstanding the difficulties enumerated and the unknown factors involved, which seem unsurmountable in any large field experiment, the data are considered significant. The degree of significance can only be determined through a statistical analysis which will be presented in the next section of this paper.

Statistical Analysis of the Data from the Previous Experiment

As indicated above, the experiment was begun April 20, 1933, by making diggings on three plots within a fenced area. The grubs

found were derived from eggs laid during July and early August, 1932. These plots were later used for separate experiments. Plot A was heavily inoculated with nematodes; B served as a control and C was lightly inoculated.

The average grubs per plot equalled:

A — 95.3; B — 78.7; C — 87.5

The first question which may be asked is whether or not it is reasonable to assume that the three plots were so chosen as to be random samples of the same general population. Working with the individual samples (10 for each plot) we find the

Variance between plots is 690 with 2 degrees of freedom
Variance within plots is 692 with 27 degrees of freedom

The ratio approximates 1.0 where 3.4 would be necessary for significance. The plots, within the errors of random sampling, may consequently be considered alike in grub population.

The nematodes were inoculated into Plot A May 23 and Plots A and C May 29. Grubs were found infected at stakes June 12, but the nematodes had not spread to "between the stakes."

The data obtained in June show:

Heavily inoculated Plot A at stakes had	12.6 grubs
Lightly inoculated Plot C at stakes had	11.7 grubs
Untreated control Plot B had	20.2 grubs

The points in between the stakes for Plots A and C which evidently correspond to the control show respectively 17.2 and 20.2 grubs. It is evident from the numbers of grubs and the percentages of infected individuals that Plots A and C at the stakes are identical. The same may be said for the points between the stakes and the control plot.

To decide whether or not the amount by which the grub population was lower at the stakes A and C is significant; is the mean 12.3 significantly less than the mean 19.2? Working with the individual diggings, we find the

Variance between the plots is 585 with 1 degree of freedom
Variance within the plots is 60 with 47 degrees of freedom

The ratio $\frac{\text{between}}{\text{within}}$ approximates 9.7 where 4.0 would be significant. It thus appears that the nematodes had significantly reduced the grub population close to the area in which they were planted, the amount of the reduction being perhaps 40 per cent. Interestingly enough, a slightly but not significantly greater number of grubs was found parasitized in the lightly infected Plot C than in Plot A.

The September 28, 1933, diggings followed just after the laying of the eggs by the 1933 crop of adult beetles. One would expect that since the beetles came from all the surrounding territory to lay their eggs the larvæ from them would be evenly distributed over all of the plots. The evidence indicates that this is the case since there is no significant difference between the average numbers of grubs for any of the plots whether at or between the stakes.

The variance between the plots is 463 with 4 degrees of freedom
The variance within the plots is 469 with 94 degrees of freedom

The ratio approximates 1.0 which is clearly not significant.* It is of further interest that the nematodes have at this time spread over both the A and C plots.

May 14, 1934, the following spring, revealed a drop in the grub population from an average of 32 per plot to 27.6 per plot, or 12 per cent. Grubs infected with nematodes have now appeared in the untreated Plot B. Besides the diggings on this plot, examinations were made on the so-called neutral areas surrounding the plots. All but the neutral area between A and B showed invasion by the nematodes. The whole area became covered with this parasite of the Japanese beetle. The greatest drop in the numbers of beetle grubs was obtained in Plot B, from 34.9 to 19.7 per square foot. This drop would certainly not be due to the parasites, if for no other reason than

* It might possibly be argued that the points in Plot C at stakes and between stakes do not have as many grubs as the control Plot B. Testing this difference gives:

Variance between Plots B and C is 580 with 1 degree of freedom
Variance within Plots B and C is 288 with 45 degrees of freedom

A ratio of 2.0 is obtained where 4.0 is necessary for significance. This comparison simply supports the more general one.

that they have only just invaded this area. Temperature experiments also indicate that until the warmer soil temperatures of middle May, the activity of the nematodes is at a low ebb. The significant differences in the drop of the grubs in Plot B as contrasted with Plot A must therefore be attributed to other causes. The most immediately significant fact for the study of the action of the nematode parasite under natural conditions is that Plot A commences the spring, which is the beginning of the active period for the nematodes, with a considerably larger population of grubs than the other plots.

By June 9, 1934, the grubs had dropped in Plot A at the stakes from 33 to 11, a difference of 22, and between the stakes from 27 to 12, a difference of 15. In Plot C at the stakes the grubs dropped from 21 to 6, a difference of 15, and between the stakes from 22 to 9, a difference of 13. The untreated Plot B dropped from 20 to 13, a difference of 7. The drop in the grub population of the treated plots was over twice that in the so-called control plot. This control has now become invaded with parasites and therefore can no longer be regarded as an uninfected control, since part of this drop may also be due to the nematodes. The difference between the drop in the grub population of Plot A, as a whole (18.4 ± 1.08) and Plot B, the control, (7.1 ± 2.36) is 11.3 ± 2.59, and the difference between A plus C (17.4 ± 0.98) and the control (7.1 ± 2.36) is 10.3 ± 2.55. These differences are 4 times their standard errors and are consequently significant. The conclusion that there is a distinct drop in grubs due to the nematode parasite thus appears justified. The numbers of grubs found parasitized by the nematodes have increased.

The September 26, 1934, diggings represent the distribution of the larvæ from the adults of the July and early August flight. Throughout all of the plots there has been a marked decline in their numbers over the 1933 year. All of the area has now become infected with the parasites. The numbers of grubs are now approximately 5 per digging as against approximately 100 or more which were present in the fall of 1932. The question may now be asked whether this infected area has less grubs than a corresponding area in the same locality which has not yet become infected with the nematodes. One hundred and ten samplings from such an area were made, 1025 grubs were found, or 9.3 per digging. In 214 samples from Plots A, B, and

C 1145 grubs or 5.4 per digging were found. The differences are significant since variance between the groups is 1176, while that within the groups is 47, a ratio of 1 to 25 where a ratio of 1 to 3.8 would be significant. It would thus appear that the grubs of the Japanese beetles are distinctly less in the area parasitized by the nematodes than in the uninoculated area. This fact is supported by more data obtained during the October 24, 1934, diggings.

The General Trend of the Japanese Beetle Infestation in the Experimental Area

The general trend of the population curve of the beetle grubs is rapidly progressing downward in all of the plots. The grub population of the fall of 1932 seems to be at least 10 per cent higher than that observed on April 20, 1933, but supposing they were the same, the change would be that observed on Tables IX and X. This analysis* of these data, for the causes contributory to the reduction in the grub population, shows that the only really significant variable is the year in which the census was taken. Time, which produces the tremendous drop in numbers of grubs from the peak of 95 per square foot in 1932 to 3 per square foot in 1934, is the significant variable. Since the grubs represent eggs from the adult beetles and since these within an infested area presumably distribute themselves at random, it is likely that the character of the plot influences the number of eggs laid upon it very little,—and this is what is found,—the plot population of grubs in the fall is not significantly different from plot to plot. It is in the spring and early summer that the nematodes destroy the grubs as shown above.

The influence of the nematodes is not widespread as yet. It seems to take them quite a while to invade adjoining fields. While, as shown above, it significantly reduces the grubs in its own area, it does not to the same extent influence the July–August flight of beetles coming from the much larger area surrounding these relatively small plots. We must at present also regard as important other factors, such as birds,

* Since the observational data for the different classes are unequal in number the method used in this analysis is that of Yates, F., *J. Agric. Sci.*, *23*, 108, as cited by Snedecor, George W., in Analysis of Variance, 1934, Collegiate Press, Ames, Iowa, page 96.

TABLE IX

Trend of Grub Population in Experimental Plots

	Plot A			Plot B			Plot C			Total		
Fall	No. of samples	Grubs	$(Grubs)^2$	No. of samples	Grubs	$(Grubs)^2$	No. of samples	Grubs	$(Grubs)^2$	No. of samples	Grubs	$(Grubs)^2$
1932	10	935	97304	10	787	67669	10	875	83025	30	2615	247998
	Mean	95.3			78.7			87.5			261.5	
1933	52	1845	96575	10	349	14739	37	977	36171	99	3171	147485
	Mean	35.4			34.9			26.4			96.7	
1934	150	837	12203	20	58	402	44	250	2904	214	1145	15509
	Mean	5.6			2.9			5.7			14.2	
Total	212	3635	206082	40	1194	82810	91	2102	122100	343	6931	410992
	Mean	17.1			29.8			23.1			20.2	
Sum of means		136.3			116.5			119.6			372.4	

$$\text{Mean } \frac{372.4}{9} = 41.4$$

Source of variation	Degrees of freedom	Sum of squares	Variance
Total	342	270986	
Between	8	198881	24860
Within	334	72105	215.88 × .058406

$$\frac{1/10 + 1/52 + 1/150 + 1/10 + 1/10 + 1/20 + 1/10 + 1/37 + 1/44}{9} = \frac{.525652}{9} = .058406$$

Sum of squares

TABLE X

Analysis of Contributory Causes of Variation in Grub Population

Sources of variation	Degrees of freedom	Sum of squares	Variance
Between means of 9 classes..................	8	10755.1	
Between means of plots.....................	2	67.3	33.6
Between means of dates.....................	2	10560.9	5280
Interaction..................................	4	126.9	31.7
Experimental error........................	334		12.6

moles, and probably many as yet unidentified, which have reduced the numbers of grubs following the successive yearly flights. It is comforting to realize that enemies and possibly other factors (climatic) tend to hold in check such an overgrowing population. It would be of interest to know accurately what each may be and what contribution each makes to the whole control problem. In the present work all that can be said is that the nematode parasite was one such factor, and produced a distinct reduction in Japanese beetle larvæ.

SUMMARY

The small field plots inoculated with *Neoaplectana* in 1931 were studied through 1934. These plots were each periodically infested with a definite number of grubs and examined from time to time for parasitized material. The soil was frequently tested for the presence of larval nematodes in the second-stage, which is the only form capable of a free-living existence. Accurate yearly records were kept of the adult emergence from these plots. The evidence obtained showed that the parasite had permanently established itself and produced a high mortality. Large field experiments were also executed and two methods for the introduction of the nematodes were practiced. One method may be defined as surface introduction by spraying; the other subsurface introduction by burying. The spraying method, to date, has not yielded encouraging results. The subsurface method of introduction, however, yielded significant results. The nematodes became established, produced a high mortality, spread over the entire experimental area and later to the surrounding field. No pronounced difference was noted in the results obtained between the heavily and lightly inoculated plots.

A statistical analysis of the experiment, started in the spring of 1933, showed that the plots within the errors of random sampling were alike in population. Later, during the same spring, the introduced nematodes significantly reduced the grub population close to the area in which they were planted, the amount of the reduction being perhaps 40 per cent. In the autumn of 1933, a fresh crop of beetle larvæ showed a population decline, but no significant difference was noted between the average number of grubs in any of the plots. In May 1934 a further drop in the population, not ascribed to nema-

todes, occurred in all of the plots. By June, however, the drop in the treated plots was over twice that in the control plot. Since by that time the control also became invaded by the parasites, part of the drop in this plot might have been due to them. The difference, therefore, between the treated and untreated areas is probably greater than indicated. The difference between one inoculated plot and the control and the difference between two inoculated plots and the control constitute differences of over 4 times their standard errors and are consequently significant. During September, 1934, a fresh crop of beetle larvæ showed a further marked decline in population. The parasites at this time were spread all over the experimental area. A test made between this parasitized area and the surrounding uninoculated field showed that the grubs had been distinctly reduced within the former.

Unfortunately it is impossible to maintain a constant, heavy population over a period of years within a given territory. Birds, moles, climate, and unknown factors are undoubtedly responsible for this instability, which complicates any experimental procedure.

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THE DISTRIBUTION OF SWINE INFLUENZA VIRUS IN SWINE

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Swine influenza is a disease of complex etiology. It is caused by the concerted action of a filtrable virus and the bacterium, *Hemophilus influenzae suis*; and neither agent alone is capable of inducing the disease (1).

While the virus of swine influenza has been found regularly in mixtures of lung and bronchial lymph nodes from infected pigs (1 and 2), it was not demonstrated, in a small number of earlier unpublished experiments, in either the spleen or heart blood by swine inoculation. Furthermore the virus was innocuous when administered intramuscularly to swine (3) and produced influenza only when it gained entrance by way of the respiratory tract. These facts are in accord with Waldmann's conception of it as a pneumotropic virus (4).

However, certain features of the clinical and pathological pictures exhibited by swine suffering an acute influenza suggest a generalized or septicemic infection rather than one in which the etiological agents (1) are limited to the respiratory tract. The prostration of infected swine is more extreme than might be expected from the amount of pneumonia encountered at autopsy, and the accompanying leukopenia (2) suggests the possibility of the presence of the infectious agents in the blood stream. At autopsy pathological alterations are encountered outside the respiratory tract: The cervical, bronchial and mesenteric lymph nodes are frequently enlarged and edematous, the spleen is usually swollen and engorged and the mucosa of the colon is congested and sometimes edematous (2). It was known from bacteriological studies that, except in fatal cases, *H. influenzae suis* was seldom encountered outside the respiratory tracts of swine ill of influenza (5). This organism could thus not be held directly accountable and it seemed possible that the filtrable virus might be responsible for the features of swine influenza suggesting a generalized infection.

The experiments reported in the present paper were conducted in an effort to determine whether the swine influenza virus was limited strictly to the respiratory tract or whether it became generalized during the course of the disease. The observation by Andrewes, Laidlaw and Smith (6) that the swine influenza virus is pathogenic for white mice has been utilized in this study of the distribution of virus in influenza-infected swine.

EXPERIMENTAL

The 8 swine used in the present experiments were infected by intranasal inoculation with glycerolated swine influenza virus mixed with a small amount of a culture of *H. influenzae suis* (2). All developed typical swine influenza and were killed with chloroform on either the 3rd or 4th day following inoculation. Portions of the organs to be tested for the presence of virus were removed at autopsy with sterile instruments. They were then ground with sand and physiological salt solution was added to make an approximately 5 per cent suspension. The suspensions were allowed to sediment for 10 minutes before the supernatant fluid was decanted to be used in inoculating mice. The tracheal exudate was scraped from the opened trachea with a sterile spatula and was prepared as an approximately 5 per cent suspension by shaking in a flask containing glass beads and physiological saline. Blood was obtained from the swine at autopsy by pipette from the seared heart. It was defibrinated with a wire whip and used undiluted in inoculating the test mice.

The white mice used in testing for the presence of virus in the various organ suspensions were inoculated intranasally, while under ether narcosis, as previously described (7). They were kept under observation for 4 days and the survivors were then chloroformed and examined for the presence of the characteristic pneumonia caused by the swine influenza virus (6 and 7). Some of the mice, especially those receiving tracheal exudate and lung, succumbed to the swine influenza virus infection on the 3rd or 4th day. Three mice were inoculated with each suspension in most cases. The results are summarized in Table I.

DISCUSSION

As shown by the data in Table I, swine influenza virus was present in the lungs, tracheal exudate and turbinates of all swine tested. It was demonstrated in only two instances in tissues outside the respiratory tract. One of each group of 3 mice inoculated with suspensions of the bronchial lymph nodes of Swine 1539 and Swine 1574 developed a scant influenzal pneumonia. To be certain that the lesions in these 2 mice were due to swine influenza virus their lungs were used to infect other mice in series; these mice all developed typical and extensive

TABLE I

The Distribution of Swine Influenza Virus in Swine

Swine No.	Extent of influenzal lung lesions produced in mice by swine tissues and materials tested											
	Lung	Tracheal exudate	Turbinates	Bronchial lymph nodes	Defibrinated blood	Blood serum	Spleen	Liver	Kidney	Mesenteric lymph nodes	Colon mucosa	Brain
1536	1, 2, 2*	2, 1, 2	2, 1, 0	0, 0, 0	0, 0, 0		0, 0, 0			0, 0, 0	0, 0, 0	
1532	2, 2, 2	4, 4, 4	1, 1, 1	0, 0, 0	0, 0, 0		0, 0, 0	0, 0, 0	0, 0, 0			0, 0, 0
1542		3, 2, 2	1, 1, 2	0, 0, 0	0, 0, 0		0, 0, 0	0, 0, 0	0, 0, 0			0, 0, 0
1539	2, 1, 3	3, 3, 3	3, 3, 1	1, 0, 0	0, 0, 0		0, 0, 0	0, 0, 0	0, 0, 0			0, 0, 0
1563	4, 3, 1	4, 4, 4	2, 2, 4	0, 0, 0	0, 0, 0		0, 0, 0	0, 0, 0	0, 0, 0			
1574	3, 4, 2	4, 2, 3	1, 1, 1	1, 0, 0	0, 0, 0		0, 0, 0	0, 0, 0	0, 0, 0			
1579-B	3, 1	1, 2	4, 3	0, 0		0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0
1573	2, 3	3, 2	1, 0	0, 0		0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0

* 0 = Mouse with no detectable influenzal lung lesions postmortem.
1 = Mouse with influenzal pneumonia involving upwards to 1/4 of lung at postmortem.
2 = " " " " " from 1/4 to 1/2 of lung at postmortem.
3 = " " " " " " 1/2 to 3/4 " " " "
4 = " " " " " " 3/4 to all of lung at postmortem. Mice showing lung lesions to this extent usually died.

influenzal pneumonias. The bronchial lymph nodes of 2 of the 8 swine examined thus contained swine influenza virus but, as judged by mouse inoculation, in very low concentration. No virus was detected in the spleens, livers, kidneys, mesenteric lymph nodes, colon mucosae, brains or blood of any of the swine studied.

Mice inoculated intranasally with either the fresh defibrinated swine blood or swine serum exhibited a picture at postmortem which deserves special comment. Their lungs contained pale grey areas of consolidation that were similar in distribution to the lesions caused by the swine influenza virus. The possibility was at first entertained that these pneumonic areas might represent unusual virus reactions. However, all attempts to transmit virus serially in mice from such lesions were unsuccessful, the lungs of mice of the first serial transfer proving normal at autopsy.

No evidence was obtained to indicate that the swine influenza virus was generally distributed throughout the bodies of any of the 8 swine studied. It was confined to the respiratory tracts of 6 of the animals and in the remaining 2 to the respiratory tracts and the regional lymph nodes. The virus evidently has a strong affinity for the respiratory tract and exerts its specific effect there. Those features of swine influenza suggesting a generalized or septicemic infection appear, therefore, to be secondary effects of the localized respiratory tract disease.

SUMMARY

Swine influenza virus was found to be regularly present in the turbinates, tracheal exudate and lungs of infected swine but not in the spleens, livers, kidneys, mesenteric lymph nodes, colon mucosae, brains or blood. It was present in low concentration in the bronchial lymph nodes of 2 out of 8 animals. This localization of the virus in swine accords with its classification as a pneumotropic virus.

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INFECTIOUS FIBROMA OF RABBITS

III. THE SERIAL TRANSMISSION OF VIRUS MYXOMATOSUM IN COTTONTAIL RABBITS, AND CROSS-IMMUNITY TESTS WITH THE FIBROMA VIRUS

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In a previous paper (1) it was suggested, on the basis of immunological similarities between the viruses of infectious fibroma and infectious myxoma, that passage of myxoma virus through cottontail rabbits (genus *Sylvilagus*) might yield fibroma virus just as passage of variola virus through calves supposedly yields vaccinia virus. The use of cottontail rabbits to effect this hypothetical transformation was suggested by the fact that the fibroma virus was originally obtained from a naturally occurring growth in one of these animals (2).

The susceptibility of the cottontail rabbit to infectious myxoma is not established to judge from the literature on the subject. Moses (3) has stated that the wild rabbits of Brazil are insusceptible to experimental infection with *Virus myxomatosum* except in rare instances and Hobbs (4) and Hyde and Gardner (5) were unable to infect our native cottontail rabbits with it. The writer, in 3 attempts to infect cottontail rabbits by subcutaneous administration of *Virus myxomatosum*, obtained 1 doubtful infection. In this rabbit a transitory thickening of the epidermis and subcutaneous tissue developed at the site of injection 16 days after inoculation (1). It seemed likely that if any hope of establishing *Virus myxomatosum* in cottontail rabbits was to be entertained, a route of inoculation other than subcutaneous should be employed.

Attempted Infection by the Intracerebral Route

A cottontail rabbit was inoculated intracerebrally with 0.1 cc. of a dilute suspension of testicular myxoma virus. The animal exhibited no signs of illness

and was sacrificed on the 9th day. The brain, which showed no macroscopic lesions, was removed and used in preparing an approximately 10 per cent suspension. A cottontail rabbit was inoculated intracerebrally with 0.1 cc. of this suspension and in addition was injected subcutaneously with 2 cc. and intraperitoneally with 8 cc. of the suspension. A domestic rabbit was inoculated subcutaneously with 1 cc. and intratesticularly with 0.5 cc. of the suspension. The domestic rabbit died of characteristic myxoma on the 11th day, while the cottontail rabbit developed no illness and was sacrificed on the 9th day. A 10 per cent suspension of its brain was prepared and injected into a cottontail rabbit and a domestic rabbit as in the previous experiment. No evidence of myxoma appeared in the domestic rabbit and consequently no further cerebral serial passages through cottontail rabbits were attempted.

From this experiment it was apparent that *Virus myxomatosum* survived for 9 days in the brain of a cottontail rabbit and was then transmissible to a laboratory rabbit, but it probably did not increase in amount since the brain of even the second serial passage cottontail rabbit failed to infect a domestic rabbit. This route of inoculation was obviously unsatisfactory in any attempt to modify the virus by prolonged serial passage.

Infection of Cottontail Rabbits by Intratesticular Inoculation

Because of the facility with which the fibroma virus infects domestic rabbits when inoculated intratesticularly, it was decided to try this route of inoculation in infecting cottontail rabbits with *Virus myxomatosum*. It was found that regular and satisfactory infections could be obtained by testicular inoculation supplemented by simultaneous subcutaneous inoculation. In all, fifteen cottontail rabbits have been infected in this manner and two by subcutaneous inoculation alone. Most of the cottontail rabbits used in these experiments were purchased in Kansas but a few trapped in the neighborhood of the laboratory were also used. No naturally immune animals were encountered.

Course of the Disease.—The clinical picture of the disease induced in cottontail rabbits by *Virus myxomatosum* proved to be very different from that seen in domestic rabbits. The incubation period was long, varying from 6 to 12 days. The disease was an entirely local process. The first evidence of infection in all instances was a slight swelling of the inoculated testicle. Usually the subcutaneous tissue at the site of injection remained negative although rarely a small

firm tumor developed. The inoculated testicle, after swelling had begun, often increased rapidly in size and became very firm, the swelling occasionally being accompanied by edema of the scrotum. The animals, however, showed no evidence of generalized illness and in special no myxomatous swellings of the eyelids nose, ears, or anus. None died, but 10 were sacrificed from 11 to 19 days after inoculation. The remaining 7 made uneventful recoveries, and it is believed that all 17 would have survived. The inoculated testicle frequently reached a size 2, and sometimes even 3 times that of the uninoculated testicle. This enlargement persisted for an indefinite period but in most instances retrogression had begun within 25 days following inoculation. Late in the course of the infection, when the scrotal edema had subsided, the inoculated testicle was frequently irregularly nodular.

Pathology.—The pathological picture in cottontail rabbits autopsied 11 to 19 days following infection was quite constant. Usually no lesion was present at the site of subcutaneous inoculation, though rarely a small tumor was encountered; firm, pinkish white, edematous and giving, on cut section, the impression of a fibroma. The inoculated testicle, in addition to being enlarged, was injected and varied in color from a pale pink to a deep purplish red. On cut section it was firm and moist and frequently white and fibromatous in appearance even though the surface of the testicle had appeared injected. The epididymis, sometimes relatively more enlarged than the testicle, was usually white or pinkish white in color, nodular, and cut as though fibrous. The scrotum, when involved, was thickened and its walls were diffusely infiltrated with a gelatinous exudate.

Only one subcutaneous tumor has been examined histologically. It had begun to retrogress at the time the animal bearing it was autopsied. The overlying epithelium was normal in appearance, and no cytoplasmic inclusions were observed. The main mass of the tumor had been composed of widely spaced large stellate connective tissue cells but these, at the time of examination, were degenerating and stained but faintly pink with phloxin-methylene blue. Pink-staining collagen fibrils, coagulated lymph, and many round cells filled the spaces between the degenerating connective tissue cells.

Four myxomatous cottontail rabbit testicles have been examined histologically. All presented similar pictures. There was a marked proliferation of connective tissue cells in the interstitium, and mitotic figures in some sections were plentiful. The arrangement of the cells varied; in some it was so loose and the individual cells so large and isolated that the appearance was that of a myxomatous infiltration. In other sections the cells were definitely of the young connective tissue type and formed compact whorls about the seminiferous tubules. In some portions of all sections necrotic seminiferous tubules were seen. This necrosis was probably secondary to pressure exerted by the rapidly proliferating interstitial tissue. Nests of round cells were present in all sections and, in some, large areas of the interstitium were densely infiltrated with this type of cell. No cytoplasmic inclusions were observed in epithelial cells in either the testicle or epididymis.

TABLE I

Passage of Virus myxomatosum Serially through Cottontail Rabbits

Passage No. and date	Rabbit No.	Infection with *Virus myxomatosum*: Supernatant of a 5 per cent suspension of	Infection with *Virus myxomatosum*: Dosage and route of inoculation	Result
1 10/31/32	DR* 4-72	Subcutaneous lesion DR 4-81 (1:50 dilution)	1 cc. s.c.† and 0.2 cc. i.t.†	Died, 11th day; typical myxoma
	CR* 4-94	Subcutaneous lesion DR 4-81	2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged when killed on 17th day
2 11/17/32	DR 5-09	Testicle CR 4-94	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 10th day; typical myxoma
	CR 5-08		2 cc. s.c.	Myxomatous tumor at site of inoculation measuring 4 x 5 x 0.75 cm.; animal killed on 18th day
3 12/ 5/32	DR 5-30	Subcutaneous lesion CR 5-08	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 11th day; typical myxoma
	CR 5-32		2 cc. s.c. and 0.5 cc. i.t.	Questionable subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged when killed on 14th day
	CR 5-33		2 cc. s.c.	Moderate subcutaneous reaction by 16th day; complete and uneventful recovery
4 12/19/32	DR 5-53	Testicle CR 5-32	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 10th day; typical myxoma
	CR 5-39		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged when killed on 19th day
	CR 5-42		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged; allowed to recover

5 1/ 7/33	DR 5-78	Testicle CR 5-39	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 15th day; typical but slow myxoma
	CR 5-43		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged when killed on 16th day
6 1/23/33	DR 6-06	Testicle CR 5-43	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 7th day; typical myxoma
	CR 6-02		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged when killed on 14th day
	CR 6-03		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged; complete and uneventful recovery
7 2/ 6/33	DR 5-93	Testicle CR 6-02	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 12th day; typical myxoma
	DR 5-68		0.5 cc. s.c. and 0.1 cc. i.t.	Died, 12th day; typical myxoma
	CR 5-98		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged when killed on 15th day
	CR 6-04		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged; complete and uneventful recovery
8 2/21/33	DR 6-43	Testicle CR 5-98	0.5 cc. s.c. and 0.1 cc. i.t.	Killed, 10th day; typical myxoma
	CR 6-45		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged and firm when killed on 15th day
	CR 6-34		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged; complete and uneventful recovery

* DR = domestic rabbit. CR = cottontail rabbit.

† s.c. = subcutaneously. i.t. = intratesticularly into one testicle.

TABLE I—*Concluded*

Passage No. and date	Rabbit No.	Infection with *Virus myxomatosum*: Supernatant of a 5 per cent suspension of	Infection with *Virus myxomatosum*: Dosage and route of inoculation	Result
9 3/ 8/33	DR 6-56	Testicle CR 6-45	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 11th day; typical myxoma
	CR 5-99		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged when killed on 11th day
	CR 6-32		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged; complete and uneventful recovery
10 3/20/33	DR 6-64	Testicle CR 5-99	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 8th day; typical myxoma
	CR 6-35		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged when killed on 14th day
	CR 6-00		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle slightly enlarged; complete and uneventful recovery
11 4/3/33	DR 7-09	Testicle CR 6-35	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 6th day; myxoma, probably complicated by intercurrent infection

Serial Passage of Virus myxomatosum through Cottontail Rabbits

The 17 animals furnishing the basis for the foregoing description of *Virus myxomatosum* infection in cottontail rabbits were part of an experiment in which an attempt was made to determine whether the virus would be modified by serial passage in this species.

Virus myxomatosum has been submitted to 10 serial cottontail rabbit passages over a period of 140 days. The inoculated testicle was used as a source of virus for each succeeding serial passage except the third when tissue from the subcutaneous lesion was utilized. The virus was tested at each passage by inoculation into domestic rabbits to detect whatever attenuating influence cottontail rabbit passage might exert upon it. In both the cottontail and the domestic rabbit infections only one testicle was inoculated. A record of the passage experiment is outlined in Table I.

Consideration of the data presented in Table I indicates that passage of *Virus myxomatosum* serially through cottontail rabbits did not attenuate it for domestic rabbits. Nothing to suggest conversion of *Virus myxomatosum* into the virus of infectious fibroma was revealed by the procedure. In the experiments recorded in Table I animals to be used as a source of virus were sacrificed on from the 11th to the 19th day following inoculation. From other experiments not recorded in this table, it is known that *Virus myxomatosum* persists in the infected testicles of cottontail rabbits and remains fully virulent for domestic rabbits for at least 21 days. In one instance it could not be demonstrated by animal inoculation after 32 days.

Immunological Relationship of Infectious Myxoma of Cottontail Rabbits to Infectious Fibroma.—

The sera from 6 cottontail rabbits recovered from infection with *Virus myxomatosum* have been tested for neutralizing properties against this virus. Myxoma virus from glycerolated domestic rabbit testicles was used for the experiment. It was prepared in 5 per cent suspension in 0.9 per cent NaCl solution and then centrifuged. The supernatant fluid after decantation was diluted 1:25 and 0.5 cc. of it was mixed with 1.5 cc. of each sample of cottontail rabbit serum under test. For controls, mixtures of 0.5 cc. of the same dilution of infectious suspension and 1.5 cc. quantities of normal cottontail rabbit serum were prepared. All of the mixtures were stored overnight (17 hours) prior to subcutaneous inoculation into domestic rabbits.

The results of the experiment were consistent in that all serum samples from myxoma-recovered cottontail rabbits possessed some neutralizing properties for *Virus myxomatosum.* The 2 control rabbits died in 10 and 11 days. 3 of the rabbits receiving mixtures containing convalescent serum died of characteristic infectious myxoma in 17, 19, and 34 days respectively. 1 rabbit, after an incubation period of 17 days, developed what appeared to be a mild myxoma and was found subsequently to have become immunized to *Virus myxomatosum.* The remaining 2 rabbits showed no evidence of illness and 1 of these tested later was found to be fully susceptible. 3 of the sera in the amounts used thus afforded some protection against *Virus myxomatosum* but failed to prevent fatal infection, 1 protected sufficiently well to prevent death while 2 protected completely.

Three of these sera were tested further for their ability to neutralize the virus of infectious fibroma by using 3 parts of serum to 1 part of 5 per cent testicular fibroma virus suspension. All 3 neutralized fibroma virus completely when the mixtures were tested by subcutaneous inoculation into domestic rabbits. The cottontail rabbits furnishing the 3 serum samples were inoculated subcutaneously and intratesticularly with fibroma virus, of proven infectivity by both routes for a control cottontail rabbit, and were found to be completely resistant to infection.

Immunological Relationship of Infectious Myxoma of Domestic Rabbits to Infectious Fibroma.—In an earlier paper (1) it was recorded that a single domestic rabbit upon recovery from an attack of myxoma induced by infection with an almost neutral serum-virus mixture was not only resistant to infection with the virus of infectious fibroma but also yielded a serum which neutralized both the fibroma and myxoma viruses. The exact proportions of neutralizing serum and virus necessary to produce non-fatal myxoma infections in domestic rabbits are difficult to ascertain. Most of the mixtures tried are found to contain either too much or too little serum, in which cases, respectively, the injected animal either acquires no illness and no immunity or develops myxoma and succumbs. However, out of a number of attempts, 3 other domestic rabbits have been given non-fatal attacks of myxoma by inoculation with almost neutral serum-virus mixtures.

These 3 animals were found immune to fibroma[1] and their sera capable of neutralizing both the fibroma and myxoma viruses. These experiments indicate that domestic rabbits, as well as cottontail rabbits, not only become resistant to fibroma virus following infection with *Virus myxomatosum* but also develop antibodies capable of neutralizing fibroma virus.

DISCUSSION AND SUMMARY

In the experiments presented, *Virus myxomatosum* was observed to produce only a localized fibromatous or myxomatous orchitis when injected into the testicles of cottontail rabbits. This type of disease was quite unlike the acute fatal illness which the virus caused in domestic rabbits. 10 serial passages of *Virus myxomatosum* through cottontail rabbits, covering a total elapsed time of 140 days, failed to alter its pathogenicity for domestic rabbits. Although it proved impossible to convert the myxoma virus into fibroma virus by serial passage in cottontail rabbits, it was found that these animals, recovered from myxoma, had a solid resistance to infection with the fibroma virus. Furthermore, their sera possessed neutralizing antibodies effective against the fibroma virus as well as *Virus myxomatosum*. A similar cross-immunological relationship was observed in the cases of domestic rabbits that had survived an attack of infectious myxoma.

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[1] Sir Charles Martin has kindly allowed me to refer here to his own unpublished experiments of a similar nature. He found that 5 rabbits that had survived infection induced either by contact or by conjunctival inoculation with a strain of *Virus myxomatosum* which varies in virulence from time to time were resistant to fibroma virus administered intradermally. All showed an allergic reaction 24 to 36 hours after inoculation with fibroma virus but the superficial hyperemia and swelling disappeared by the 3rd day and no fibromas developed. 5 to 8 months intervened between the recovery of these rabbits from myxomatosis and the test inoculation with fibroma virus.

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INFECTIOUS FIBROMA OF RABBITS

IV. THE INFECTION WITH VIRUS MYXOMATOSUM OF RABBITS RECOVERED FROM FIBROMA

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In experiments described in the preceding paper (1) it was shown that serial passage of *Virus myxomatosum* through cottontail rabbits did not modify its pathogenicity for domestic rabbits. Furthermore, cottontail as well as domestic rabbits were found to be resistant to infection with the fibroma virus after infection with *Virus myxomatosum*, and their blood sera were effective in neutralizing both the myxoma and fibroma viruses. So far as these immunological data go, they suggest the identity of the fibroma and myxoma viruses.

However, as disclosed by earlier work (2), the immunological relationship in the opposite direction was quite different. Although domestic rabbits which had recovered from the fibroma exhibited an increased resistance to infection with *Virus myxomatosum*, which is ordinarily fatal, their sera possessed no demonstrable neutralizing properties for this virus. Furthermore, the clinical and pathological pictures of the fibroma and myxoma infections in rabbits were so different that the identity of the two viruses seemed improbable. The experiments to be described in the present paper were conducted in an effort to determine the nature of the resistance to myxoma induced in domestic rabbits by infection with the fibroma virus.

Infection of Fibroma-Recovered Domestic Rabbits with Virus myxomatosum

Fourteen out of 15 fibroma-recovered domestic rabbits were reported earlier (2) to have survived infection with *Virus myxomatosum*. Since then, the number of fibroma-recovered domestic rabbits inoculated

with amounts of *Virus myxomatosum* that would ordinarily be fatal has been increased to 62 and of these 59 have survived the infection. Control animals inoculated each time have regularly succumbed. Of more than 150 normal domestic rabbits infected during this period with the same strain of *Virus myxomatosum* none survived. This uniform fatality of *Virus myxomatosum* for domestic rabbits is in accord with the experience of others investigating the disease. The minimum time for the establishment of a state of resistance to fatal infection with *Virus myxomatosum* is not known. That it is something less than a fortnight is indicated by the fact that 2 rabbits in the series were found to be resistant to fatal myxoma infection 14 days after inoculation with the fibroma virus. Rabbits tested for resistance to myxoma as late as 100 days after their primary fibroma infection proved resistant. In the cases of the 3 fibroma-recovered rabbits above mentioned that died of myxoma following inoculation with *Virus myxomatosum* 24, 35, and 56 days had elapsed between the primary inoculation with fibroma virus and the inoculation with *Virus myxomatosum*.

Repeated injections of fibroma virus failed to enhance the resistance of rabbits to myxoma or to establish antibodies neutralizing *Virus myxomatosum* in their sera. 2 rabbits that had received 2 injections and 1 that had received 4 injections of fibroma virus subcutaneously and intratesticularly were found to be no more resistant to *Virus myxomatosum* than animals receiving but a single injection. Furthermore, the sera of these 3 rabbits possessed no demonstrable neutralizing properties for *Virus myxomatosum*.

The wide time range over which infection with fibroma virus exerts its protective influence against fatal infection with *Virus myxomatosum* (from 14 to 100 days) seems to eliminate the possibility that the increased resistance is of a non-specific nature. This is further indicated by the fact that the mere injection of fibroma virus, even in large dosage, confers no protection; actual fibromatous growth is necessary. For instance, domestic rabbits injected intraperitoneally even with very large amounts of fibroma virus develop no growths and are subsequently still fully susceptible to fatal infection with *Virus myxomatosum*; on the other hand, relatively much smaller amounts of fibroma virus given subcutaneously or intratesticularly regularly result in growths and the establishment of resistance to infectious myxoma.

The Clinical and Pathological Picture of Infectious Myxoma in Fibroma-Recovered Animals.—As was pointed out previously (2), fibroma-recovered rabbits were but rarely completely resistant to *Virus myxomatosum*. Most of the animals developed myxoma in an abortive form, and the lesions, though characteristic of the disease, were limited to the formation of a localized myxomatous growth if the inoculation had been subcutaneous, or to a myxomatous orchitis if the inoculation had been intratesticular. Sometimes these local processes were accompanied by mild conjunctivitis and a purulent type of rhinitis which were transient. Myxomatous swelling of the eyelids, nose, ears, and genito-anal region developed rarely and the picture presented was that of typical acute infectious myxoma, differing from it, however, in that this condition was not fatal. In many instances in which fibroma-recovered rabbits were inoculated both subcutaneously and intratesticularly with *Virus myxomatosum*, myxomatous lesions developed only in the testicle. In respect to the general clinical picture, fibroma-recovered domestic rabbits reacted to infection with *Virus myxomatosum* in much the same manner as did normal cottontail rabbits (1). It appears that preliminary infection with fibroma virus induces in the highly susceptible domestic rabbit a resistance to *Virus myxomatosum* similar in degree to that exhibited naturally by the cottontail rabbit.

The gross and histopathological characters of the local lesions developing in the testicles or subcutaneous tissues at the site of *Virus myxomatosum* inoculation in fibroma-recovered domestic rabbits were similar to those at corresponding sites in fully susceptible domestic rabbits. The healing process seen only in resistant rabbits was characterized by a marked infiltration of the local lesions with round cells. Cytoplasmic acidophilic inclusions were present in epithelial cells of the epididymis of the inoculated testicle and in those of the epidermis overlying local growths in the subcutaneous tissue in resistant rabbits. These were identical in appearance with the inclusions seen in similar cells of fully susceptible rabbits.

Recovery of Virus myxomatosum from the Local Myxomatous Lesions Induced in Fibroma-Recovered Rabbits and Its Passage in Series through Such Animals.—Preliminary experiments showed that when fibroma-recovered domestic rabbits were inoculated subcutaneously or intratesticularly with *Virus myxomatosum* and developed only a local myxomatous lesion, myxoma virus, fully pathogenic for normal rabbits could be recovered from such lesions even as late as 16 days after inoculation. The local lesions by this time were often regressing. This rendered likely the possibility that the virus might prove serially transmissible in such animals.

In order to study the relationship of fibroma to myxoma virus, and because of the possibility of altering the pathogenic properties of

Virus myxomatosum, it seemed advisable to attempt the serial passage of this virus through fibroma-recovered rabbits. Further, the question first raised by Rivers (3) of whether *Virus myxomatosum* is a single virus or composed of more than one virus might be answered. For instance the immunological relationships between the fibroma and the myxoma viruses, outlined earlier in this paper, were in accord with the possibility that *Virus myxomatosum* might be composed of fibroma virus and some other perhaps hitherto unknown virus. If such were the case, it could easily be understood why *Virus myxomatosum* would immunize completely against the fibroma virus, one of its components, while the fibroma virus, being but one part of *Virus myxomatosum*, gives correspondingly only partial immunization.

In the serial passage of *Virus myxomatosum* through fibroma-recovered rabbits at least one normal control rabbit was inoculated at each passage to detect any change in the character of the disease induced by the virus. Male rabbits were used and, in the cases of the resistant rabbits, the preliminary inoculation with fibroma virus had been made subcutaneously and into one testicle. In inoculating such animals subsequently with *Virus myxomatosum* the other testicle and a new subcutaneous site were chosen. Fresh *Virus myxomatosum* from the subcutaneous lesion of a rabbit dead of the disease was used in starting the experiment. At each passage the infected testicle from a resistant rabbit was used in inoculating animals of the succeeding passage. Usually the testicle was removed, under ether anesthesia, on from the 10th to the 12th day following inoculation, thus allowing the animal furnishing the virus to recover and complete its record in the experiment. In the first 2 passages, the animals serving as the source of passage virus were killed. No virus for passage was taken from a resistant rabbit until the normal control animal had died. *Virus myxomatosum* was passed in this manner through fibroma-recovered rabbits for 8 serial passages at which time the experiment was discontinued. An outline of the experiment is presented in Table I.

Consideration of the data given in Table I reveals that *Virus myxomatosum* was readily transmissible in series through the testicles of fibroma-recovered rabbits. Its pathogenic properties, as judged by inoculation into normal domestic rabbits, were unchanged by such passage. None of the fibroma-recovered rabbits used in the experiment died and most showed only a localized testicular myxomatosis.

Rous, McMaster, and Hudack (4) have shown that living cells protect viruses associated with them from the neutralizing effect of immune serum. The possibility, suggested by this work, that living

cells in the inoculum administered at each serial passage served to shield the virus from neutralization in the resistant animals and thus perpetuate it from passage to passage was shown not to be a factor; myxoma virus from infected testicles stored for 2 months in 50 per cent glycerol at refrigerator temperature induced localized myxomatous orchitis when administered intratesticularly to fibroma-recovered rabbits and virus was demonstrable in such local lesions.

The Disappearance of Virus myxomatosum from the Site of Inoculation in Immune Rabbits.—An animal wholly immune to a virus not only yields a specific neutralizing blood serum and is completely refractory to reinfection but in addition, when reinoculated, is capable of rendering injected virus rapidly non-demonstrable. The failure to recover virus from the sites of inoculation in immunized animals has been reported repeatedly.

Kraus, Keller, and Clairmont (5) demonstrated that rabies virus could not be got from the brains of immunized rabbits 5 days following inoculation, and Kraus and Doerr (6) found that while fowl plague virus was still demonstrable in the brains of immunized geese 6 hours following inoculation, this was not the case 18 hours after injection. Levaditi and Nicolau (7) observed that vaccinia virus inoculated into the brain of an immunized rabbit could not be demonstrated 2 hours following injection. Andrewes (8) showed that Virus III inoculated into the testicles of immunized rabbits was not to be recovered 2 hours after injection and Nicolau and Kopciowska (9) made a similar observation regarding herpes virus introduced into the brains of immunized rabbits. Smith (10) noted that while vaccinia virus persisted in the circulation of a susceptible rabbit for as long as 8 days following intravenous infection, it disappeared from the circulation of an immune rabbit within 4 to 6 hours.

The following experiment was performed in an attempt to demonstrate a similar phenomenon in animals immune to *Virus myxomatosum.*

Three male rabbits were made resistant to infectious myxoma by a preliminary subcutaneous infection with fibroma virus. They were next submitted to a subcutaneous inoculation of *Virus myxomatosum.* A local myxomatous lesion developed in the subcutaneous tissue at the site of inoculation. Following regression of the lesion, the sera of these rabbits contained demonstrable neutralizing antibodies for *Virus myxomatosum* and the animals were deemed immune. They were then inoculated subcutaneously and into each testicle with a suspension of *Virus myxomatosum* from glycerolated infected testicles. As controls, a normal and a fibroma-recovered rabbit received the virus in a similar manner. The normal

TABLE I

Passage of Virus myxomatosum Serially through Fibroma-Recovered Rabbits

Passage No. and date	Domestic Rabbit No.	Previous treatment	Infection with *Virus myxomatosum*: Supernatant of a 5 per cent suspension testicle Rabbit No.	Infection with *Virus myxomatosum*: Dosage and route of inoculation	Result
1 10/31/32	4-72	None (control)		1 cc. s.c.* and 0.2 cc. i.t.*	Died, 11th day; typical myxoma
	4-56	Subcutaneous fibroma	1:50 dilution 5 per cent suspension of subcutaneous myxomatous lesion Rabbit 4-81	1 cc. s.c.	No general symptoms; slight local subcutaneous lesion; recovered
	4-65	Subcutaneous and testicular fibroma		1 cc. s.c. and 0.2 cc. i.t.	No general symptoms; inoculated testicle moderately enlarged; recovered
	4-74	Subcutaneous and testicular fibroma		1 cc. s.c. and 0.2 cc. i.t.	No general symptoms; inoculated testicle moderately enlarged when killed on 11th day
2 11/11/32	5-04	None (control)	4-74	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 8th day; typical myxoma
	4-66	None (control)		0.5 cc. s.c. and 0.1 cc. i.t.	Died, 8th day; typical myxoma
	4-71	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; slight local subcutaneous lesion; inoculated testicle moderately enlarged; recovered
	4-61	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; slight local subcutaneous lesion; inoculated testicle moderately enlarged when killed on 11th day

3 11/22/32	5-13	None (control)		0.5 cc. s.c. and 0.1 cc. i.t.	Died, 9th day; typical myxoma
	4-87	None (control)		0.5 cc. s.c. and 0.1 cc. i.t.	Died, 9th day; typical myxoma
	4-77	Subcutaneous fibroma	4-61	0.5 cc. s.c.	No local lesion or illness
	4-78	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; no subcutaneous lesion; inoculated testicle moderately enlarged when removed on 10th day;† recovered
4 12/ 2/32	4-44	None (control)		0.5 cc. s.c.	Died, 10th day; typical myxoma
	4-68	Subcutaneous and testicular fibroma	4-78	0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; no subcutaneous lesion; inoculated testicle moderately enlarged when removed on 11th day; recovered
5 12/13/32	5-28	None (control)		0.5 cc. s.c. and 0.1 cc. i.t.	Died, 8th day; typical myxoma
	5-20	Subcutaneous and testicular fibroma	4-68	0.5 cc. s.c. and 0.1 cc. i.t.	Completely negative
	5-21	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; no subcutaneous lesion; inoculated testicle moderately enlarged when removed on 11th day; recovered
6 12/24/32	5-27	None (control)		0.5 cc. s.c. and 0.1 cc. i.t.	Died, 11th day; typical myxoma
	5-23	Subcutaneous and testicular fibroma	5-21	0.5 cc. s.c. and 0.1 cc. i.t.	Clinical picture characteristic of myxoma; recovered
	5-16	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; no subcutaneous lesion; inoculated testicle slightly enlarged and firm when removed on 11th day; recovered

* s.c. = subcutaneously; i.t. = intratesticularly into one testicle.

† All operative procedures were conducted under full ether anesthesia.

TABLE I—*Concluded*

Passage No. and date	Domestic Rabbit No.	Previous treatment	Infection with *Virus myxomatosum*: Supernatant of a 5 per cent suspension testicle Rabbit No.	Infection with *Virus myxomatosum*: Dosage and route of inoculation	Result
7 1/ 4/33	5-77	None (control)	5-16	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 8th day; typical myxoma
	5-24	Subcutaneous fibroma		0.5 cc. s.c.	No general symptoms; moderate local subcutaneous myxomatous lesion
	5-17	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	Completely negative
	5-50	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; no subcutaneous lesion; inoculated testicle greatly enlarged when removed on 11th day; recovered
8 1/15/33	5-71	None (control)	5-50	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 12th day; typical myxoma
	5-22	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; scant local subcutaneous lesion; inoculated testicle slightly enlarged and firm when killed on 12th day
9 1/27/33	5-72	None	5-22	0.5 cc. s.c.	Died, 13th day; typical myxoma

control rabbit developed typical myxomatosis and died on the 9th day. The fibroma-recovered control developed localized subcutaneous and testicular myxomata, a transient conjunctivitis, and survived. The 3 myxoma-immune animals remained normal. A testicle was removed, under ether anesthesia, from each of the control rabbits 20 hours following infection, and from the myxoma-immune animals 20, 48, 72, and 96 hours following inoculation. The testicles were ground in a mortar, suspended in physiological saline, and a portion of each suspension thus prepared was inoculated subcutaneously and intratesticularly into rabbits to test for the presence of *Virus myxomatosum*. *Virus myxomatosum*, inducing typical infectious myxoma fatal in 9 and 10 days, respectively, was demonstrable in the testicles removed at the end of 20 hours from the normal and fibroma-recovered control rabbits. The testicles removed 20, 48, 72, and 96 hours following inoculation from the myxoma-immune rabbits were free from *Virus myxomatosum* demonstrable by rabbit inoculation.

The results of this experiment suggest that, as in other virus diseases, virus inoculated into the tissues of rabbits immune to infectious myxoma is promptly destroyed or rendered non-demonstrable.

The Failure of Virus myxomatosum to Invade the Blood Stream of Fibroma-Recovered Rabbits.—In infectious myxoma of rabbits the etiological virus invades the blood stream and is regularly found there throughout the later course of the disease (11 and 12). It seemed of interest to determine whether it was similarly present in the blood stream of fibroma-recovered rabbits after inoculation with *Virus myxomatosum*.

Three fibroma-recovered rabbits that developed a localized myxomatous orchitis after intratesticular inoculation with *Virus myxomatosum* were bled from the ear vein on the 2nd, 4th, 7th, and one on the 11th day after infection. Serum from each of these bleedings failed to produce infectious myxoma in test rabbits to which it was administered subcutaneously in 3 cc. amounts. Similar amounts of serum obtained from non-resistant rabbits, infected with myxoma, from the 7th day post-infection to death regularly produced infectious myxoma in test rabbits to which it was similarly administered.

These experiments indicated that *Virus myxomatosum* did not invade the blood stream in fibroma-recovered rabbits as it did in fully susceptible animals. They did not, however, shed light on the actual mechanism by which *Virus myxomatosum* is restrained to a localized and relatively benign infection in fibroma-recovered rabbits. This will be considered in more detail later.

TABLE II

The Time of Appearance of Myxoma Neutralizing Antibodies in the Blood Serum of Fibroma-Recovered Rabbits Infected with Virus myxomatosum

Serum from Rabbit No.	Drawn, days after inoculation with *Virus myxomatosum*	Effect of subcutaneous injection of mixture of 0.5 cc. 1:25 dilution of *Virus myxomatosum** + serum		
		Amount of serum in mixture	Injected Rabbit No.	Result
		cc.		
	Fibroma-convalescent (before myxoma)	3	8-99	Died, 15 days
	2 days	3	8-91	Died, 17 "
	4 "	3	8-85	Died, 17 "
8-65	7 "	3	8-81	No illness
	10 "	3	9-04	No illness
	15 "	1.5	9-41	No illness
	46 "	1.5	9-39	No illness
	Fibroma-convalescent (before myxoma)	3	9-00	Died, 13 days
	Fibroma-convalescent (before myxoma)	3	9-54	Died, 11 "
	2 days	3	8-83	Died, 17 "
8-66	4 "	3	8-89	Died, 11 "
	7 "	3	8-84	No illness
	10 "	3	9-03	No illness
	15 "	3	9-55	No illness
	46 "	1.5	9-43	No illness
	Fibroma-convalescent (before myxoma)	3	8-98	Died, 12 days
	Fibroma-convalescent (before myxoma)	5	9-52	Died, 12 "
	2 days	3	8-86	Died, 22 "
	2 "	5	9-40	Died, 13 "
	4 "	3	9-05	Died, 18 "
	4 "	5	9-46	Died, 16 "
8-70	7 "	3	8-88	Died, 17 "
	7 "	5	9-42	Died, 13 "
	10 "	3	9-02	Died, 13 "
	10 "	5	9-57	Died, 25 " (probably not of myxoma)
	15 "	5	9-55	No illness
	46 "	5	9-53-A	No illness
	Fibroma-convalescent (before myxoma)	3	11-03	Died, 18 days
10-57	7 days	3	11-00	Died, 15 "
	17 "	3	11-24	No illness

* *Virus myxomatosum* = supernatant of a 5 per cent suspension of glycerolated testicle and subcutaneous lesion from rabbit dead of infectious myxoma.

TABLE II—*Concluded*

Serum from Rabbit No.	Drawn, days after inoculation with *Virus myxomatosum*	Effect of subcutaneous injection of mixture of 0.5 cc. 1:25 dilution of *Virus myxomatosum** + serum		
		Amount of serum in mixture	Injected Rabbit No.	Result
		cc.		
	Fibroma-convalescent (before myxoma)	3	11-05	Died, 15 days
10-58	7 days	3	10-99	No illness
	17 "	3	11-20	No illness
	Fibroma-convalescent (before myxoma)	3	11-01	Died, 16 days
10-69	7 days	3	10-98	No illness
	17 "	3	11-21	No illness
	Fibroma-convalescent (before myxoma)	3	11-22	Died, 17 days
10-70	7 days	3	10-97	Died, 13 "
	17 "	3	11-23	No illness
8-55	Normal	3	8-96	Died, 14 days
14	Normal	5	9-53-B	Died, 10 "
14	Normal	3	10-35	Died, 11 "
10-74	Normal	3	11-07	Died, 16 "
10-74	Normal	3	11-19	Died, 20 "

The Time of Appearance of Myxoma-Neutralizing Antibodies in the Blood Serum of Fibroma-Recovered Rabbits Infected with Virus myxomatosum.—In the preceding section it was noted that *Virus myxomatosum* could not be detected in the serum of myxoma-infected fibroma-recovered rabbits. It seemed possible that the failure of the virus to generalize in these animals might be due to their prompt generation of virus-neutralizing antibodies. Such an occurrence would aid in explaining the benign and localized nature of their myxoma infections. Therefore, the time of appearance of myxoma-neutralizing antibodies in the blood serum of infected fibroma-recovered rabbits was investigated.

A series of rabbits was bled following recovery from infection with the fibroma virus. They were then inoculated either subcutaneously or intratesticularly, or by both routes, with *Virus myxomatosum* in amounts large enough to kill all control animals. Serum was obtained from 3 of the rabbits on the 2nd, 4th, 7th, 10th, 15th, and 46th days and from the remaining 4 on the 7th and 17th days following infection with *Virus myxomatosum*. This serum, together with that obtained prior to their infection, was then tested for its ability to neutralize *Virus myxomatosum*.

The neutralization tests were conducted in the usual fashion. The serum-virus mixtures were set up to contain 0.5 cc. of a 1:25 dilution of the supernatant of a 5 per cent suspension of glycerolated subcutaneous and testicular myxoma lesion (the equivalent of 1 mg. of infectious tissue) mixed with the amount of serum being tested, usually 3 cc. The mixtures were stored overnight (17 hours) in the refrigerator prior to injection subcutaneously into the test rabbits. Rabbits receiving mixtures which were neutral developed no evidence of infectious myxoma and survived. Those receiving mixtures in which the serum did not neutralize the virus came down with the typical disease and died in from 11 to 20 days following inoculation. These rather long survival periods are believed to be the result of the relatively small amounts of virus employed in the mixtures. The amount of virus used was, however, sufficient to kill all control rabbits. The results of the neutralization experiments are outlined in Table II.

The data recorded in Table II reveal that of the serum samples obtained prior to the 7th day following infection with *Virus myxomatosum* none neutralized the virus. Of 7 of the samples obtained on the 7th day following infection, however, 4 neutralized the virus completely. Of the remaining 3 rabbits, the serum of 1 failed to neutralize virus on the 10th day but did on the 15th day, while the other 2 both neutralized on the 17th day post-infection. An attempt to determine a possible relationship between the time of appearance of neutralizing antibodies and the severity of the myxoma infection leads to the impression that the promptness of antibody reaction was determined by the severity of the infection, rather than that the severity of the infection was determined by the promptness with which antibodies were produced. 3 of the 4 rabbits whose serum contained neutralizing antibodies as early as the 7th day post-infection developed either a coryza or a conjunctivitis in addition to myxomatous swellings at sites of inoculation, while 2 of the 3 rabbits in which the appearance of demonstrable antibodies was delayed until later than the 7th day post-infection exhibited no evidence of generalizing infection. The exceptional animal in each group is sufficient to indicate that any

attempt to correlate the speed of production of antibodies with the extent and severity of the disease in so small a group of experimental animals is hazardous. The fact remains, nevertheless, that fibroma-recovered rabbits produce antibodies capable of neutralizing *Virus myxomatosum* during an attack of the modified infectious myxoma that they develop. The sera of fully susceptible rabbits infected with myxoma virus at no time contains neutralizing antibodies and, as was pointed out in the preceding section, is rich in virus from the 7th day post-infection to death. The time of appearance of virus neutralizing antibodies in the sera of resistant rabbits thus approximately coincides with that at which the virus ordinarily generalizes in susceptible animals. It seems possible that this fortunate coincidence of events may be at least partially accountable for the apparent resistance of fibroma-recovered rabbits to *Virus myxomatosum.*

DISCUSSION

The transmissibility of *Virus myxomatosum* in series through fibroma-recovered rabbits without alteration of its disease-producing properties, in contrast with its failure even to survive in the tissues of myxoma-immune rabbits, is of importance as far as reaching a decision concerning the identity of the fibroma virus with *Virus myxomatosum.* The generally accepted criterion for considering two viruses identical is an immunological one. Animals recovered from infection with each virus should not only resist infection with the other virus but their sera should neutralize it. They should, furthermore, be capable of inactivating or destroying the other virus when it is introduced into an ordinarily susceptible tissue. So far as the immunological relationship between the fibroma virus and *Virus myxomatosum* is concerned these criteria of complete cross-immunity are not fulfilled. Even though infection of a rabbit with fibroma virus is known to establish in that animal a state of enhanced resistance to *Virus myxomatosum*, the fibroma-recovered rabbit is usually not completely refractory to myxoma infection as evidenced by the development of a local myxomatous lesion at the site of inoculation. Furthermore injection of *Virus myxomatosum* into a fibroma-recovered rabbit does not result in the destruction of the injected virus. On the contrary, the virus actually multiplies and can be passed indefinitely in series through

such resistant animals. Finally, serum from a fibroma-recovered rabbit, though neutralizing the fibroma virus, is without effect on *Virus myxomatosum.* It is plain that the two viruses are not identical.

The results of cross-protection and cross-neutralization experiments can be best explained on the basis of a partial duplication of the antigenic components comprising the two viruses. It was shown in the preceding paper (1) that rabbits recovered from *Virus myxomatosum* infection were immune to the fibroma virus and their sera capable of neutralizing that virus. This indicated that *Virus myxomatosum* contained antigenic components essential to the production of a complete fibroma virus immunity. On this basis, the incomplete protection of rabbits in the reverse direction might be interpreted as indicating that the fibroma virus is antigenically only a partial replica of *Virus myxomatosum.* The antigenic components comprising fibroma virus and common also to the myxoma virus are sufficient to establish in fibroma-infected rabbits a state of resistance to myxoma, but, because they represent only partially the antigenic composition of *Virus myxomatosum,* this resistance is not the complete immunity conferred reciprocally by two identical viruses.

The experimental data presented are considered to support the view, evident also from the clinical and pathological data, that, while perhaps antigenically and genetically closely related, *Virus myxomatosum* and fibroma virus are different infectious agents.

SUMMARY

The serial passage of *Virus myxomatosum* through fibroma-recovered domestic rabbits did not alter its pathogenic properties. Fully virulent *Virus myxomatosum* persisted in the inoculated testicle of fibroma-recovered rabbits for at least 16 days following inoculation. Virus injected into the testicles of myxoma-immune domestic rabbits, on the other hand, was promptly rendered non-demonstrable. The failure of fibroma-recovered domestic rabbits to destroy injected *Virus myxomatosum* and the absence from their sera of neutralizing antibodies effective against *Virus myxomatosum* are considered to be evidence against the identity of the fibroma and myxoma viruses. The rapidity with which fibroma-recovered rabbits develop neutralizing

antibodies following infection with *Virus myxomatosum* is considered to be a possible factor in their acquired resistance.

It is believed on the basis of all the evidence that infectious fibroma of rabbits is a definite disease entity and not merely a mild and non-fatal form of infectious myxoma.

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STAPHYLOCOCCI ASSOCIATED WITH MASTITIS*

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Mastitis associated with staphylococci, although less common than streptococcic mastitis, may occasionally become very acute. In most herds the infection is confined to a single animal, generally a heifer or cow recently introduced or an individual shortly after parturition. The affected animal may completely recover, or a partial or serious injury to the diseased quarter may remain, or death may occur. Occasionally the udder becomes gangrenous, resulting in the sloughing of the diseased parts.

Guillebeau[1] found that staphylococci were responsible for severe udder inflammations and that some types liquefied gelatin. Savage[2] studied five cases of staphylococcic mastitis. From his material he obtained 22 strains, of which 16 attacked mannite and 19 liquefied gelatin. Evans[3] reported that, in milk drawn from the udder, 58.8 per cent of the samples contained micrococci. Both pathogenic and non-virulent varieties were present in the skin, which were of the same types as those found in milk. The hemolytic aureus types appeared to be more pathogenic than the non-hemolytic albus strains. Jones[4] considered that next to streptococci, the micrococci were more frequently involved in udder disease than any other microörganisms. He intimated that prognosis in mastitis was more favorable with a staphylococcic infection than one caused by streptococci. The fermentative characteristics of 28 strains were given. Of these, 23 liquefied gelatin at 22° C. and 21 attacked glucose, lactose, saccharose and mannite. In three cows a chromogenic micrococcus was obtained from abscesses of the udder. This condition usually occurred in cows recently introduced into the herd. Carpenter[5] inoculated 2 cc of a 24-hour broth culture of non-hemolytic *Staphylococcus aureus*, obtained from a case

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of mastitis, into the udders of three heifers and two cows. Four animals reacted severely, showing a high temperature with loss of appetite for at least 36 hours following the inoculation. Ten days later, one cow died from septicemia resulting from the infection of the udder and at autopsy staphylococci were isolated from all the tissues of the body. In three animals abscesses appeared in the udders. Minett *et al.*[6] reported six cases of mastitis attributed to staphylococci and suggested that this type of mastitis was more severe than the usual form caused by streptococci, since two out of six cases died. In the majority of these, the infection occurred shortly after parturition. The microörganism on agar, solid serum or potato produced colonies which were dirty white or pale fawn in color and coagulated litmus milk with production of acid. Gelatin was liquefied after incubation, for two or three weeks at 15 to 18° C. Acid was produced in lactose, glucose, saccharose and mannite. Broth cultures were usually fatal to mice. Plasteridge *et al.*[7] found that in about 10 per cent of their cases of udder inflammation staphylococci were responsible. From the results of their observations it would appear that mastitis staphylococci attack mannite while the more harmless varieties do not.

During the past 17 years, the late Dr. F. S. Jones and one of us (R. B. L.) have occasionally encountered severe cases of staphylococcic mastitis in dairy cows and a certain number seem worthy of special mention. In 1918, Jones reported in some detail the clinical and bacteriological findings of a case of mastitis attributed to staphylococci. Since then a culture from this cow (C 60) has been carried in stock and is included in this publication so the case will be presented again. At the onset of mastitis the left hind quarter was firm, swollen and tender to manipulation. The secretion was thick, yellowish white, and contained 2,172,000 staphylococci per cc. The acute attack persisted for about ten days before the infection subsided. In 1932, we had occasion to examine a herd of 130 cows in which two animals had previously died as a result of mastitis. In six cows showing clinical symptoms of mastitis, staphylococci were the causative pathogens. Before the outbreak subsided four cows died and three others were sold later as unfit for milk production. During the course of the disease, the affected cows were very sick, depressed and had elevated temperatures. There was inappetence, with diminution in milk.

The diseased quarters were swollen and firm, with a scanty blood-like secretion. In two cows the quarters became abscessed. Cultures of staphylococci designated as "Gray Cow 52" and "Gray Cow LF I" were obtained from individual quarters of two cows. On November 20, 1934, mastitis was observed in the right fore quarter of cow U 234. The quarter was swollen and firm with nearly a complete cessation of secretion. Two days later, mastitis was detected in the right hind quarter. The temperature was elevated and the cow appeared ill Before she was destroyed, both quarters were very firm and the skin had a bluish-red tinge. The mastitis was attributed to staphylococci and culture U 234 was recovered from the bloody exudate of the right fore quarter. Six days later, another cow in the same herd developed mastitis in the left fore quarter and died within 48 hours. Three samples of milk examined at 12-hour intervals during the course of the disease indicated that staphylococci were responsible for the infection and culture S 313 was isolated.

Since the small staphylococci are occasionally so pathogenic for cattle, it is difficult to understand why they are found in the milk of some individuals in enormous numbers without inducing death or serious inflammation of the udder.

In the daily examination of milk from three young cows over a period of six weeks, we have frequently encountered enormous numbers of typical staphylococci in the fore milk with very few organisms detectable in the remainder. The leukocyte count, the pH and the percentage of chlorides were also within normal limits. In a dilution of 1:200 of the fore milk in veal infusion blood-agar plates the count averaged between 10,000 and 60,000 microörganisms per cc with very few staphylococci present in the residual milk. Therefore this observation suggested that the growth of staphylococci under normal conditions was confined to the teat-canal, indicating that the milk in the secretory portions of the udder was not satisfactory for bacterial multiplication. It is assumed that the growth of the organisms in the udder was maintained at a low level by the action of some natural barrier present in the udder secretions.

In previous communications[8, 9] it has been shown that freshly drawn milk possesses a substance which is capable of inhibiting multiplication of bacteria for varying periods of time. It has also been proved

that the substance varies in concentration in different cows and from quarter to quarter of the same animal. This is well illustrated in the following experiment. The milk from a cow with staphylococcic mastitis was examined bacteriologically with the result that the infection was found to be confined to the left hind quarter. The organisms

FIG. 1. Petri dish containing 3 cc of mastitis milk, heated at 58° C. for 20 minutes, and incubated for 24 hours with 10 cc of veal-infusion agar to which approximately 1,000 staphylococci had been added.

were not present in the secretions from the three other quarters. When the milk from the left hind quarter was heated at 58° C. for 20 minutes (fig. 1) and then plated with 10 cc of veal infusion agar inoculated with approximately 1,000 staphylococci, no inhibition occurred. If, on the other hand, 3 cc of a mixed milk (fig. 2) from the three normal quarters was plated in a like manner, complete inhibition occurred. If 1 cc of the milk from the left hind quarter was mixed

with 2 or 3 cc of the milk from the normal quarters (fig. 3), the organisms grew without hindrance. In another experiment, when 1 cc of normal cow serum was mixed with 3 cc of milk from the normal quarters (fig. 4), it was found that the milk lost its inhibitory action with the addition of the serum.

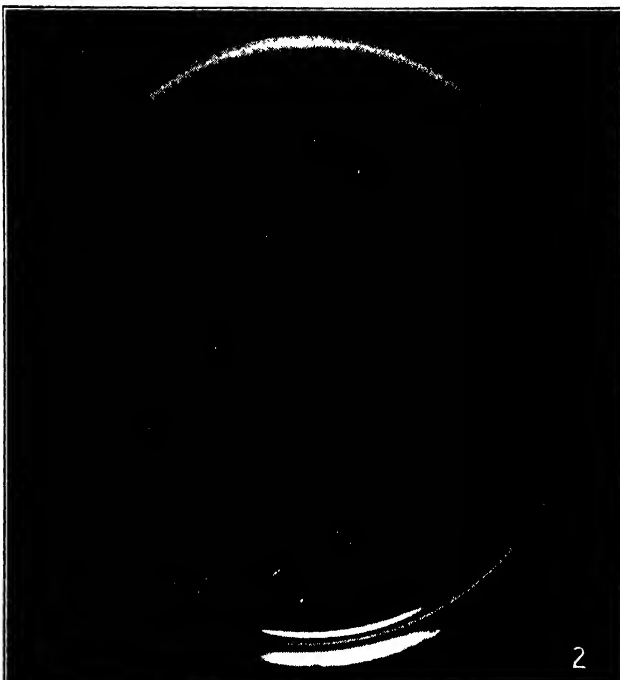

FIG. 2. Petri dish containing 3 cc of mixed milk from three normal quarters, heated at 58° C. for 20 minutes, and incubated for 24 hours with 10 cc of veal-infusion agar to which approximately 1,000 staphylococci had been added.

Recently a small staphylococcus has been recovered in pure culture from five superficial abscesses of the skin of the udder. An abscess associated with staphylococci appears as a small whitish or reddish vesicle on the outer epidermal layer of the skin. At the onset, it varies in size from a small millet seed to that of a pea. Once the abscess is opened or ruptured, it dries up and leaves a temporary, dry, scaly depression in the skin. On the other hand, the inflammatory process

may become acute, involving deeper portions of the udder, causing considerable swelling of the quarter and pain at milking. The abscess is local, for the secretions are in no way altered during an attack. There is no evidence at hand which suggests that staphylococci penetrate through the wall of the abscess and reach the secretory tissues.

FIG. 3. Petri dish containing 1 cc of mastitis milk mixed with 2 or 3 cc of milk from normal quarters, heated at 58° C. for 20 minutes, and incubated for 24 hours with 10 cc of veal-infusion agar to which approximately 1,000 staphylococci had been added.

Since in one cow a pure culture of *B. pyogenes* was recovered from a deep udder abscess, it is evident that other organisms also may be responsible for the infection of the skin of the udder.

The typical colonies of staphylococci and those associated with abscesses of the skin are small and spherical in shape. On agar plates, after 24-hour incubation, the colonies are moist, raised, and of a

grayish tint which usually changes to an orange or a light fawn color in a few days. The color characters are well defined on agar slants which have become rather dry or in milk-agar plates. Bouillon cultures, after 16 to 24 hours, are uniformly turbid, with the formation of a slight amount of soft sediment. In older cultures an adherent

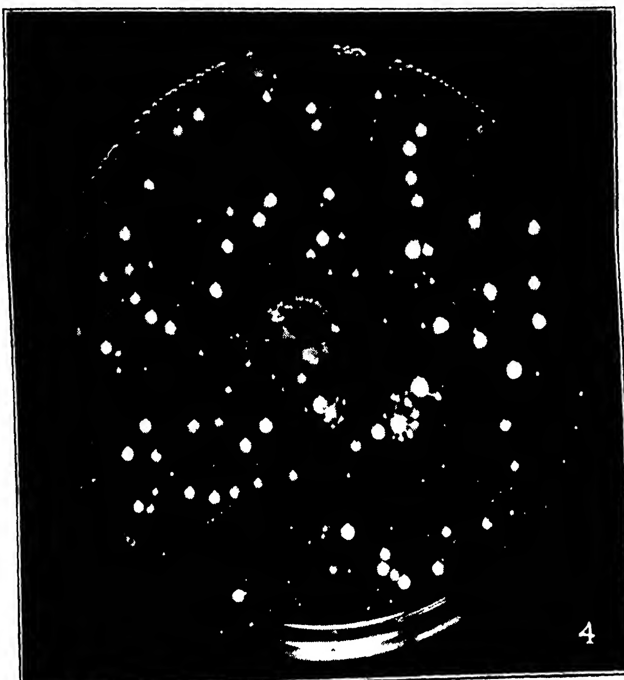

FIG. 4. Petri dish containing 1 cc of fresh, normal cow serum mixed with 3 cc of milk from normal quarters, heated at 58° C. for 20 minutes, and incubated for 24 hours with 10 cc of veal-infusion agar to which approximately 1,000 staphylococci had been added.

ring may appear around the tube at the upper surface of the broth. On fresh, sugar-free blood-agar, surface colonies show, after 24-hour incubation, slight zones of beta hemolysis which on further incubation increase in diameter. Deep colonies as a rule show little if any hemolysis, except in heavily seeded plates. The microörganisms have a tendency to clump on films or form small chains of two to four seg-

ments. The individual staphylococci, since they measure on the average less than 1μ in diameter, are smaller than the non-virulent strains. With the Gram stain the results are not constant and vary considerably with the age of the culture. If stained properly, most recently isolated strains give a Gram-positive reaction; whereas, with older cultures the reaction is less certain.

It was found that the cultures recovered from abscesses of the skin of the udder or from secretions of cows which died or had recovered from a staphylococcic mastitis were comparable in size and pigment formation. It therefore seemed of interest to determine if the cultures had other characters in common. Hucker,[10] in his studies of the parasitic and saprophytic coccaceae, developed a classification and showed that certain tests proved to be of great assistance in separating the group into subgroups and species. These tests comprised: reactions in milk, presence or absence of chromogenesis, nitrate reduction, liquefaction of gelatin and the ability to utilize ammonium salts as the sole source of nitrogen. Breed[11] studied 177 cultures of micrococci isolated from udders and classified 171 of them, according to Hucker's system, into twelve groups. Thirty-three strains were regarded as being *Micrococcus aureus* and 21 as *M. albus*. These species were apparently established in the udders, since they were isolated over a period of some weeks. This same technic, in addition to certain other tests, was here applied to our organisms. To obtain a complete description of the preparation and reactions of the reagents used by Hucker and Breed, reference to their papers may be made.

The cultural differences between the typical and a few miscellaneous strains are presented in table I.

In table I it is shown that twelve cultures of the small-type staphylococcus, isolated either from milk or udder abscesses, form a rather definite group. All strains are small in size, orange to a light fawn in color, and produce acid in dextrose, lactose, mannite and glycerin. Nitrates are reduced to nitrites and gelatin is liquefied. Of the twelve cultures studied, none were able to use ammonium phosphate as the sole source of nitrogen. Pathogenicity tests in mice demonstrated that only three strains failed to kill within 24 to 48 hours following intraperitoneal inoculations of 0.2 cc of young broth cultures. Of these, one culture was isolated by Jones in 1918 and the other two in

TABLE I

The Cultural Reactions of 20 Strains of Pathogenic and Saprophytic Staphylococci

CULTURE	SOURCE	CHROMOGENESIS	DEXTROSE	LACTOSE	MANNITE	GLYCERIN	REDUCTION OF NITRATES TO NITRITES	LIQUEFACTION OF GELATIN	AMMONIUM PHOSPHATES	HEMOLYSIS	LITMUS MILK	INTRAPERITONEAL INOCULATION OF MICE WITH 0.2 CC. OF YOUNG BROTH CULTURE
1	C 60 milk	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	–
2	Gray cow 52 milk	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	–
3	Gray cow I LF milk	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	Died 6 days
4	S 313 LF milk	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	Died
5	U 234 RF milk	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	Died
6	1932 LH abscess	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	Died
7	1932 LH milk	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	Died
8	1937 LH abscess	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	Died
9	1937 abscess B	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	Died
10	1938 RF milk	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	Died
11	1938 RH abscess	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	Died
12	1938 LF abscess	Orange to light fawn	+	+	+	+	+	+	–	–	Acid and coagulated	Died
13	1937 RF milk	Orange to light fawn	+	+	+	+	+	+	–	+	Soft curd	–
14	1932 LH milk	Orange to light fawn	+	+	+	+	+	+	–	–	Soft curd	–
15	Bottled milk	Orange to light fawn	+	–	+	+	+	+	–	+	–	–
16	1932 LF milk	Lemon yellow	+	+	+	–	+	–	+	+	Acid and coagulated	–
17	1932 LF milk	Lemon yellow	+	+	+	–	+	–	+	+	Acid and coagulated	–
18	1938 RH milk	Lemon yellow	+	+	+	+	–	–	+	+	Soft curd	–
19	1949 LH milk	Orange	+	+	–	–	+	–	–	+	–	–
20	1949 LF milk	Dirty yellow	+	+	–	–	+	+	–	–	Acid and coagulated	–

Growth in bouillon was turbid in all cases.

1932. With one culture, obtained in 1932, a mouse lived six days. It is possible that the two cultures (1 and 2) lost their infectivity by repeated transfers. It is recognized, according to Hucker's classification, that twelve out of 15 strains isolated from milk or associated with abscesses of the skin of the udder have the same cultural characteristics as *M. aureus*. Our cultures uniformly show the production of pigment on agar slants and milk plates. Cultures 13, 14 and 15 gave an atypical reaction in litmus milk and failed to kill mice. The cocci are larger than our typical strains and two produce hemolysis on blood-agar so they are not strictly comparable to the typical strains. With the remaining organisms (16, 17, 18, 19 and 20) the reactions differ in many respects. The pigment varies from light yellow or lemon yellow to orange. All attack dextrose and two fail to produce acid in mannite, while four reduce nitrates to nitrites. Three cultures can use ammonium phosphate as a source of nitrogen and, with the exception of culture 20, all fail to liquefy gelatin. In litmus milk three cultures react similarly to the typical strains; whereas, another fails to reduce litmus and one (18) produces a soft curd in milk. Four cultures are markedly hemolytic, while one shows no hemolysis. All strains fail to kill mice after 21 days. According to Hucker's classification, cultures 16 and 17 are designated as *M. varians*, 18 as *M. luteus*, 19 as *M. aurantiacus*, and 20 as *M. citreus*.

It is shown in the graph (fig. 5) that staphylococci, associated with abscess of the skin, when introduced into the udder, are capable of producing an acute severe infection presenting the same clinical manifestations of disease as occur in field cases. Cow 1932 calved for the first time on October 6, 1934. Two weeks after parturition, the hair over the udder and rear parts was clipped short in order to keep the cow as clean as possible. Before each milking, the udder was washed with a sterile cloth moistened in warm water. It was next wiped with another dry sterile cloth. Later it was noticed that small abscesses occasionally appeared on the skin over the quarters. October 31, a small abscess over the posterior ventral portion of the left hind quarter was incised and culture 6 was recovered from the exudate. From October 9, the milk was examined daily for 31 days, with the result that at times the staphylococcus count varied between 10,000 and 20,000 per cc. At different intervals during these preliminary

tests, staphylococci were frequently cultured and found to be of the large, non-pathogenic variety which does not attack mannite. November 16, culture 6 was introduced into the left hind quarter by

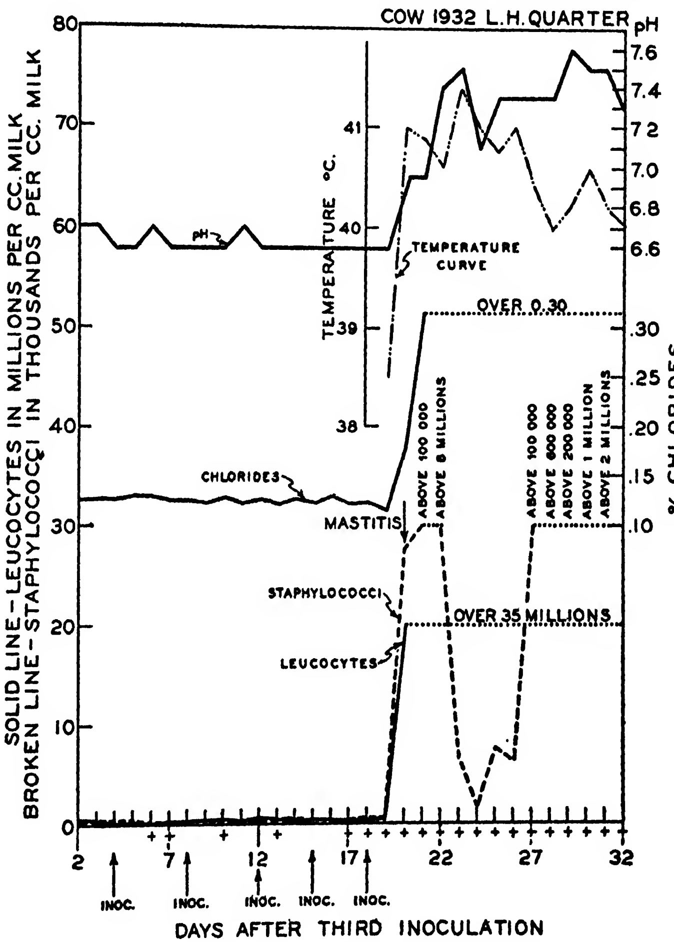

FIG. 5. Graph showing the results of the inoculation of the left hind quarter of cow 1932 with staphylococci obtained from a superficial abscess of the skin of the udder.

means of a glass rod with a small beaded end. Approximately 800 staphylococci were conveyed to the udder on this inoculation. A second injection was made ten days later, in which about 500 staphylo-

cocci were instilled into the quarter. Between the first and second inoculations the typical microörganisms were recovered on only one examination. On December 3 (6 days later), the quarter was injected with about 200 staphylococci. A fourth inoculation with approximately 900 organisms was made four days later. From then on, a series of five inoculations, spaced at 3- or 4-day intervals, was performed. In an interval of 15 days between the third and last inoculations, typical staphylococci were recognized on six different occasions. During this period, about 2,500 staphylococci were injected. On the second day following the last inoculation, a severe acute mastitis developed. The quarter was greatly swollen, congested and painful to manipulation. The secretions were scanty, yellowish and thick. The following day the temperature was 41° C. There was inappetence with diarrhea. The cow was very sick and occasionally had a severe chill. The cell count of the milk was above 35,000,000 per cc, with high points in chlorides and pH. From then on until the cow was humanely destroyed on January 4, she progressively became emaciated and weak. From the graph it is evident that the left hind quarter was severely involved. The chloride determinations were much higher than in any case of mastitis so far encountered. On the second, third and fourth days of infection, a very high peak in the number of staphylococci occurred, which later reached a low level for four days. Thereafter the organisms again appeared in the milk in enormous numbers. During the course of the disease, the temperature always was elevated and before death the udder felt cold to the touch.

DISCUSSION

In the cases studied, the saprophytic staphylococci found in the udder have been separated morphologically and culturally from the staphylococci associated with mastitis. The latter could not be differentiated from the organisms found in abscesses of the skin of the udders.

In the normal udder, the saprophytic staphylococci may thrive in enormous numbers without causing any appreciable change in the character of the secretions. They rarely, if ever, produce mastitis. With the pathogenic staphylococci the onset of the infection is very acute, with extensive swelling of the gland. The mastitis is associated

with fever and malaise, indicating a septicemia frequently terminating in the death of the animal.

It is suggested that in cases of mastitis the concentration of the inhibitory substance in the affected quarter may have been of such low potency that the multiplication of the organisms was not confined to the teat-canal but rapidly extended to the secretory tissues. On the other hand, it has been reported by other workers that frequently attacks of staphylococcic mastitis were encountered in cows immediately following parturition. In such animals it is possible that the serum passing to the udder at this period of gestation so changed the character of the colostrum or milk that the organism became well established at this time. Following parturition, the udder secretions may fail to return to normal on account of the bacterial irritation and a suitable medium for rapid growth is provided. In fatal cases or in cows with severe udder derangement the rate of introduction of the microörganisms into the udder may influence the ensuing disease. When actively growing bacteria reach the udder, multiplication may be so rapid that the inhibitory substance usually so potent for staphylococci fails to function. In udders carrying large numbers of cocci in the teat-canal the organisms may have been conveyed to the quarter in such small numbers that they were held in check.

In the skin the staphylococci may cause either small superficial abscesses or extend more deeply into the subcutaneous tissues, creating a severe local inflammation. It has been shown that these same staphylococci, when introduced into the udder, are capable of producing a very acute mastitis.

The question arises as to the incidence of udder abscesses. In many dairy herds the hair over the udder and rear quarters is clipped for sanitary purposes. Before each milking, the udder is washed with water and dried. It is possible that this procedure may remove the natural secretions of the skin which, in turn, may cause a dry, unnatural scaly condition of the epidermis which predisposes the skin to bacterial invasion.

Barber[12] and Ramsey and Tracy[13] have reported that staphylococci derived from the udders have been responsible for attacks of gastroenteritis in man. Further study will be necessary to determine whether the bovine staphylococci here described have cultural characters different from the food-poisoning strains.

CONCLUSIONS

It has been shown that certain staphylococci isolated from milk secretions of normal udders, from fatal cases of mastitis and from superficial abscesses of the skin of the udder, comprise a definite group possessing common characteristics. Freshly isolated strains are pathogenic for mice, and mastitis has been produced in a cow by small numbers of organisms from a culture obtained from a skin abscess.

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INHERITANCE AS IT AFFECTS SURVIVAL OF RATS FED A DIET DEFICIENT IN VITAMIN D

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Those familiar with experiments on diets realize that within a group of animals given the same food in like quantity, the reactions of individuals may be quite diverse. On another diet a similar group may show similar individuality but the curve of such variation may be distinctly separate from that of the first group. Experimentation in nutrition has attempted to eliminate individual variation in the animals and to emphasize the differences between given diets. In the light of the objectives sought, this attitude is commendable. Individual variation within experiments, however, seems to have a significance not to be overlooked. Individuality as a cause of variability has the same primary importance with respect to the characteristic pathology which animals develop under experimental dietary conditions as under those of nature. The food requirements of even closely related genera may often be quite distinct. From the point of view of evolution, this seems significant since the causes of such differences in food requirements are fundamental to the correlation between the spread of animals and their subsequent development.

For the study of diverse individual variations which animals may show toward a given diet, vitamin D was chosen as the major variable in our experiments. This nutrient principle is apparently a single chemical entity necessary for normal body development and maintenance of many vertebrates. The symptoms which develop in the absence of it depend somewhat upon the age of the animal. A lack of it in the young, when bones are developing actively, may lead to rickets and osteoporosis, due to improper deposition or actual extraction of calcium from growing skeletal structures. Growth is impaired or stopped; teeth are poorly calcified and often pitted. A deficiency

at puberty may be correlated with crippling and painful changes which often lead to difficulties in reproduction. Such conditions are more striking in females than in males. In the pregnant mother, excessive fetal demands for calcium and phosphorus may induce symptoms of osteomalacia. Later, the nursing mother often shows somewhat the same pathological conditions due to similar demands in the production of milk. In middle-life, osteomalacia sometimes develops if the diet is sufficiently inferior, although the changes tend to appear less rapidly than in the young. Birds show particularly severe symptoms in the form of leg-weakness which may be rapidly fatal in the young chick. Tetany often follows long-continued deprivation in some cases. Deficiency is also accompanied by reduction and degeneration of the spleen, deposition of calcium in the walls of arteries, a reduction of blood-clotting time, hemorrhage and striking change in the parathyroids. An increase in the size and number of epithelial cells of the parathyroids is followed by shrinkage and regression of the cell cords and, in some cases, a hyperplasia of the stroma. Marked keratinization then takes place. The antirachitic principle may be supplied through food or through exposure of the body to direct sunlight. In the latter case, the color of the exposed skin materially influences the amount of sunlight absorbed and hence its antirachitic effect (1).

The question which will be studied in this paper is the effect of inheritance on the utilization of vitamin D. The criterion for measuring the effect is the variation in the length of time that the animals survive when fed a diet deficient in the vitamin.

Material

Genetic research has shown that a population of animals bred at random, or nearly so, generally contains different inherited potentialities. Two individuals, though they be carefully selected are apt to deviate in their reaction to any given variable even if the experiment is well controlled. It is evident that this random differentiation would vitiate the analysis we propose. The aim is to keep the full spread of inherited differences of the initial population, but to segregate it into lines each of which has a distinctly reduced inherited variation. Close inbreeding of the brother and sister type furnishes a means by

which this end may be accomplished. If from a population of rats, we select a dozen pairs, we have a sample of the inherited potentialities of the entire group. Any given pair will be likely to contain but a limited part of the variation in the total spread of inheritance. The progeny of such a pair will tend to have only a portion of the inherited variation which their parents possessed, although considered collectively, the whole litter, if large, might well contain all of it. Each generation, so long as the inheritance of the line comes through a single pair, will consequently become more and more alike, until a time is reached when the progeny within a given generation are so closely similar that they can be considered essentially alike in respect to inheritance. Any variation which such a population shows may therefore be attributed to environment. Those familiar with the effects of such factors as linkage and balanced lethals will realize that even here difficulties may enter. However, the same procedure in inbreeding each of the sample lines originally selected at random will result in uniformity within each of the lines, but between the lines, if the selection is fortunate, the full inherited variation of the original random population will remain. The differences between such lines may be used to measure the influence which inherited variation may exert on the particular variable studied.

The experiment described here was fortunate in having available to it 16 lines so distinctly differentiated in their inheritance. We are indebted to Dr. M. R. CURTIS of the INSTITUTE FOR CANCER RESEARCH at COLUMBIA UNIVERSITY, New York, for the animals which we used to start each of our lines, and for the data from which it has been possible to obtain the survivorship of these lines when fed our complete diet (CURTIS and BULLOCK 1923; CURTIS, DUNNING and BULLOCK 1933). The sixteen strains were originally from the stock rats of four different dealers and from one European laboratory strain. When the selected pregnant females were transferred to Rockefeller Institute at Princeton, New Jersey, the different lines had been bred brother by sister for 6 to 9 generations. Three to 5 generations of further inbreeding intervened before the animals were used in the experiment. Care was taken to avoid the introduction of transmissible diseases and parasites into these new lines. Before transfer, the pregnant females were examined thoroughly and dipped to eliminate

any external parasites. After transfer, they were separated from each other by solitary isolation in different laboratories. Here the young were born and at weaning time the mothers were autopsied. Lungs were examined especially for pneumonia, feces for paratyphoid bacilli and the eggs of nematode worms. All females abnormal in any of these particulars were eliminated along with their litters. The remaining litters were then bred and the process repeated in like manner. Finally the stock was free of paratyphoids, nematodes, skin parasites and pneumonia, for a time at least. Unfortunately we missed the middle ear focus of the actinoides organism and as a result the pneumonia which it favored subsequently appeared in our stock. We could not find that its incidence had any distinct relation to the deaths in different lines whether they were subjected to the vitamin D deficient diet or to the normal one. That the disease is capable of control by the measures taken is indicated by the fact that it has been eliminated in one strain of our stock (NELSON and GOWEN 1930).

The untreated rats were maintained on the following stock ration. Bread, whole milk, fresh vegetable and yellow corn were fed on Monday, Wednesday and Friday; bread, milk and cereal on Tuesday and Thursday; bread, milk, cereal and yellow corn on Saturday. The cereal was composed of equal parts of cracked whole wheat, rolled oats and yellow cornmeal mixed with milk and cooked for 20 minutes. A special rat biscuit and water were before the animals at all times. Meat was fed once a week. Such a diet was chosen for the untreated rats because of its wide variety of foodstuffs and its ability to maintain the animals' condition.

The choice of a diet for the test animals presented many difficulties. Two diets low in vitamin D were in common use; that of MCCOLLUM, 3143 (1922), and that of STEENBOCK and BLACK, 2965 (1925). Both rather readily produce the vitamin D deficient picture in rats fed on them. The calcium-phosphorus ratio is unbalanced in both, the calcium being high and the phosphorus low. Furthermore, both diets are believed to be deficient in vitamin B_2, and also in certain minerals and iodin. Either diet, however, has in its favor the real advantage of having been widely used in other feeding experiments. Since comparability with the experiments of others seemed desirable, it was decided to use the 2965 diet in preference to any new ration which

we might devise. The proportions of this diet were 76 percent of ground yellow corn especially selected for its brightness of color, 20 percent of wheat gluten, 1 percent of sodium chloride and 3 percent of calcium carbonate. Distilled water was continuously before the animals.

The experiment was conducted in the following manner. Rats to be fed the vitamin D deficient diet were kept out of contact with direct sunlight. Females were isolated from the time that pregnancy was noted and the resulting litters were kept separate from other rats. The litters were weaned 3 weeks after birth and from then until 46 days of age, when the vitamin D deficient diet was started, they were fed on the regular stock ration. At 46 days of age, the young were put into individual screen-bottom cages 9 inches square and held $\frac{3}{4}$ of an inch above the shelf and away from direct sunlight. Food was always present in the cages. Its daily consumption was reckoned on the difference between the weight put into the cage on one day and that remaining the next. It is evident that animals could obtain some of the metal elements from the wire of their cages; other than that and their diet, it is believed that they received nothing. In the daily routine procedure, all cages were examined for feces which might have caught on the wire bottom, and shelves under the cages were cleaned. The animals were weighed once each week.

Data pertinent to the problem included litter size, initial weight at 46 days of age, maximum weight, age when maximum weight was attained, time of death and weight at death. From these items the rate of gain and absolute gain from initial to maximum weight may be calculated. The subsequent loss in weight and rate of loss until death may also be determined. The food intake was found to rise somewhat at first, then to remain quite constant until the week of death when the records show some drop. In view of the fact that differences in litter size and in weight are regarded as in part hereditable and since the aim of the experiment was to test inheritance, no adjustment in litter size and weight was made. Obviously such a procedure would have overthrown some of the objectives of the experiment. This problem is approached, however, by what is possibly a better technique in a later section of the paper.

Originally it was intended to revive some of the long-time survivors

of the diet deficient in vitamin D by giving them a normal diet and ultraviolet light. The plan was not carried far because rats, although revived in many respects by such treatment, were found to be sterile.

It is clear that although the experiment is directed at the effect of inheritance on a deficient supply of vitamin D, other more or less significant nutrients have been reduced. We should, of course, like to say that the results obtained relate entirely to vitamin D and the effects of inheritance in making this element more or less essential for the metabolism of one strain but not indispensable to a strain of different genetic constitution. This, it seems, we can not do completely, if for no reason other than our present ignorance of many essentials of nutrition. All the experiment can hope to indicate is that given a diet with certain deficiencies, vitamin D important among them, the susceptibility of different groups varied, and that a portion of this variation was attributable to inheritance. In referring to the subsequent results the term "vitamin D deficient diet" is intended to convey the broader definition. Considered from the point of view of evolution, such variations may indicate the adaptability of certain races to border zones deficient in particular environmental factors and in DAVENPORT's sense may fit races for invasion into what would otherwise be inhospitable regions.

The present paper directs attention to the variation in length of life of genetically different strains of rats fed the same vitamin D deficient diet. We may begin our study, however, by an analysis of the variation curves of the lives of all of the rats taken together. Through the courtesy of Dr. M. R. CURTIS in supplying the data, it has also been possible, for the benefit of the reader, to place on the charts life curves of rats of the same strains when fed a completely adequate diet. A total of 4981 male and 7607 female completed lives are represented in this material.

Life Curves of Rats on the Vitamin D Deficient Diet

Data from all lines were combined into a life curve representing the effects of the vitamin D deficient diet on the whole population. The total data compared with those for the human life tables are rather pitifully small, for collecting material under the conditions and requirements of the experiment is slow work. But surprisingly smooth

curves result. There are also the real advantages that the data were collected with a definite purpose in view and that all the entering variables were recorded at the time of occurrence and not at death.

Sex Differences in the Reaction to the Vitamin D Deficient Diet

Sex, of course, is an inherited character, being dependent on the chromosome distribution, although the conditions bringing it about have thus far defied exact analysis. Since a sex difference is manifest in the data, the original curve is divided into 2 curves, 1 for each sex.

TABLE 1

Survivorship Curves of Rats on Vitamin D Deficient Diets

Age Days/30	Numbers Which Survived	
	Males	Females
2	170	167
3	162	154
4	141	139
5	108	104
6	78	73
7	60	50
8	44	29
9	32	15
10	25	8
11	16	2
12	11	1
13	6	
14	5	
15	4	
16	1	

The tabulation represents survivors against duration of life, duration of life being measured as age in days divided by 30. The males in table 1 show a somewhat greater duration of life than the females.

Figure 1 gives the graph of these results. If we test the significance of differences between the sexes by comparing the specific death rates, we find that the χ^2 value for 10 classes is equal to 14.7; the P value is slightly less than 0.1 (ages of 10 and above are grouped into one class). We should have to conclude that sufficient data were not at hand to prove that there was a distinct difference between the sexes. But

the fact that a sex difference is manifest in our data in the direction of a more severe effect of the vitamin D deficiency on females than males is in line with observation on the human in puberty and afterlife and in my own unpublished results on the domestic fowl. A similar effect has been noted by BILLS and others (1931) in line test studies on rats, the males showing a slightly greater rate of healing than the females.

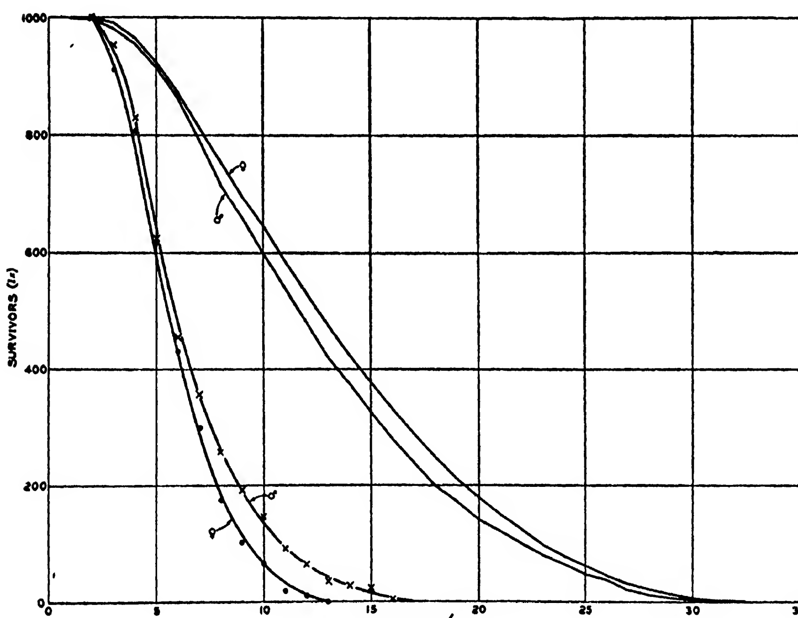

FIGURE 1.—Curves showing the survivorship plotted against age for rats on vitamin D deficient diets, left-hand curves, and like rats on a normal diet (based on CURTIS' data), right-hand curves.

Sex differences in reactions to specific agents of disease and to other agents are becoming rather well known. WRIGHT and LEWIS (1921) have shown such differences in the resistance of guinea pigs to tuberculosis. In their experiments, as in ours on vitamin deficiency, the females died more rapidly than the males. In mice the writer's experiments are showing a reversal of this effect: exposure of a population to the effects of ricin poison causes the males to die at distinctly greater rates than the females (GOWEN and SCHOTT 1933). In rat and mouse

typhoid, and mouse liver disease (*B. piliformis*), however, the sexes react alike (IRWIN 1929; SCHOTT 1932; WEBSTER 1933; GOWEN and SCHOTT 1933).

A comparison of the left- and right-hand curves in figure 1 shows that rats on a diet believed to be adequate have a distinctly longer life-span than those on the deficient diet. In the former group, 11–12 months elapses before 50 percent of the population dies, in the latter only 5–6 months intervenes. After this point, the survivors of the deficient diet begin to die off more sharply than the others and the form of the curve therefore changes. This type of curve is also found when Drosophila are subjected to starvation. Apparently when one major cause of death overshadows all others the curves tend to have an abrupt, relatively limited cycle. This suggests the destruction of a reserve substance at a given rate. The fact that the rats on the vitamin D deficient diet lived but one half as long as those on the complete diet is sufficient evidence to emphasize the importance to life of the deficiencies observed in this diet. These differences are without regard to the inheritance since the inheritance within two groups is quite similar.

The sex differences in rats on the normal diet are well marked, no question of their statistical significance being possible. They are, however, in a direction opposite that of rats on the vitamin D deficient diet, that is, the males die sooner on the normal diet than the females. Such a difference between the rats on the two diets seems significant, for while it is not possible to assert that the male and female rats are strictly not identical, the probability that the females would live longer than the males, as they do under the normal diet, becomes rather small, about 1 in 100.

Characteristics of the Curve Showing the Frequency of Death

The constants of the frequency curves of the population, variable in its heredity, have special interest when they are compared with those of the like population when the variation brought about by the heterogeneous heredity is removed. The frequencies of death against age are shown in table 2 for the population lacking vitamin D.

Figure 2 shows the frequency polygons, in which percentages of death are plotted against age for rats on normal and vitamin D deficient

diets. The two sexes are represented separately. The striking feature of these diagrams is that animals on the vitamin D deficient diet reach a much higher total death rate per unit of time than those on the normal diet. For purposes of comparison, the constants of PEARSON's frequency curves are useful in clarifying these differences. These constants are found in table 3.

The constants of table 3 show that the frequency distribution of the deaths of rats on the vitamin D deficient diet has a distinct positive skewness. The curve prescribed for these distributions is PEARSON's Type I.

TABLE 2

Frequency of Deaths of Rats on a Vitamin D Deficient Diet

Age Days/30	Numbers Which Die	
	Males	Females
2	8	13
3	21	15
4	33	35
5	30	31
6	18	23
7	16	21
8	12	14
9	7	7
10	9	6
11	5	1
12	5	1
13	1	
14	1	
15	3	
16	1	

If we compare the constants derived for animals on the vitamin D deficient diet with those for a similar group on the normal diet (CURTIS' data), we note that while the average duration of life in the latter animals is not quite twice as great as that of the former, the standard deviation is more than twice as large. The coefficient of variation consequently shows a relatively greater variation in the deaths of rats on the normal than on the vitamin D deficient diet. The skewness of both curves is within the same range. The constants of both sets of observations lead to Type I curves.

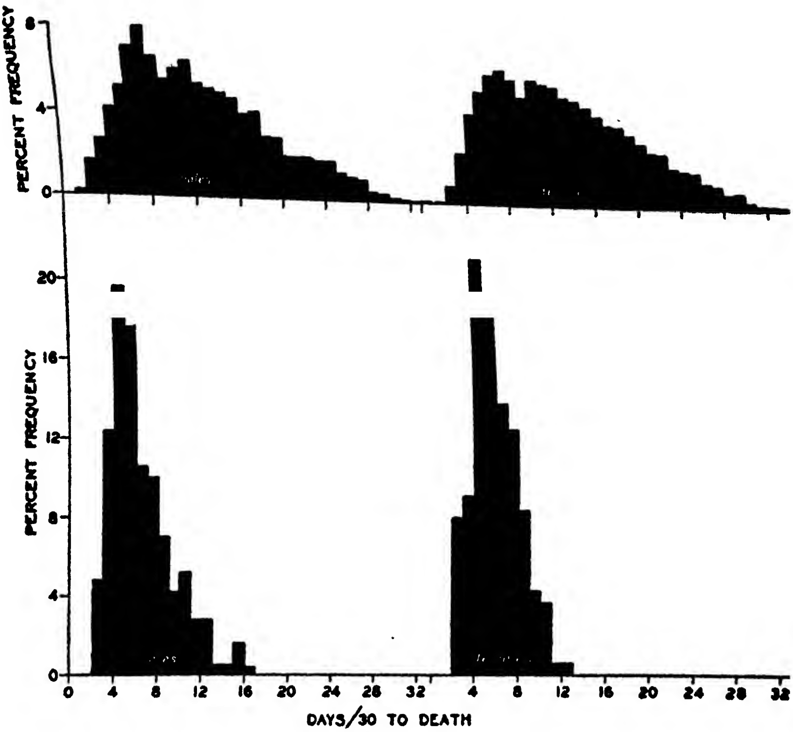

FIGURE 2.—Frequency diagrams for percentages (upper figure) of death plotted against age for rats on normal diets (derived from CURTIS' data), and on vitamin D deficient diets (lower figure).

TABLE 3

Frequency Constants for Deaths of Rats Plotted against Age, Normal and Vitamin D Deficient Diets

	Vitamin D Deficient Diet		Normal Diet (Curtis' Data)	
	Males	Females	Males	Females
Mean (days)	197.3 ±4.6	178.3 ±3.3	379.4 ±1.8	403.6 ±1.5
Standard deviation	89.0 ±3.3	63.4 ±2.3	189.9 ±1.3	198.1 ±1.1
Coefficient of variation	45.2	35.6	50.1	49.0
μ_2	8.80	4.46	40.13	43.6
μ_3	28.85	4.77	159.40	151.3
μ_4	299.22	56.13	4406.85	4840.4
β_1	1.22± .27	0.26± .11	0.39± .02	0.28± .01
β_2	3.86± .48	2.82± .25	2.74± .04	2.55± .02
Skewness	1.27± .38	0.41± .10	0.77± .04	0.70± .03
Type of curve	I	I	I	I

Characteristics of the Frequency Distributions of Deaths of the Sexes When Each Genetically Separăte Group Is Centered on Its Mean

We may turn to the question, what are the characteristics of the death curves when the hereditable variation in the susceptibility of the animals is removed? Inbreeding tends to purify a race in the sense of making one animal genetically like the other. The strains of rats used in these experiments had all been inbred nine or more generations by brother and sister matings. They should, therefore, be quite pure from the point of view of inheritance. The variation which remains within each strain may be looked upon as that due to

TABLE 4

Constants of the Distributions of Deaths for Rats on Vitamin D Deficient Diet When Each Genetically Separate Group Is Centered on Its Mean

	Males		Females	
Mean	−0.0	days	−0.2	days
Standard deviation	56.1±2.0	days	42.16 ±.6	days
μ_2	3.49	days	2.02	days
μ_3	1 98	days	0.36	days
μ_4	52.85	days	13.39	days
β_1	0.092± .186	days	0.015±.010	days
β_2	4.187±2.43	days	3.036±.365	days
Skewness %	0.096± .083	days	0.061±.064	days

external environmental conditions. Any differences between the strains, on the other hand, may be assigned to differences in the genetic constitution of the strains. By centering the variability curve of each strain on its mean, we eliminate the variation due to heredity and are in a position to examine the variation due to environment. The constants of the distributions of deaths for male and female rats on the vitamin D deficient diet are seen in table 4.

When each genetically separate group is centered on its mean, the standard deviations have become markedly reduced. This shows that the strain differences played a real part in the resistance of the population to the lack of vitamin D. The frequency distributions have become more symmetrical, the Beta constants do not differ

significantly from the values of the normal curve $\beta_1 = 0$ $\beta_2 = 3$. The skewness of both the male and female groups has been eliminated with the removal of the major inheritance differences. The frequency curves are now essentially symmetrical.

The constants indicate that when the heterogeneity introduced by differences in inheritance is removed, Gaussian curves will approximate the survival curve of the rats. This fact has particular significance to studies of the potencies and effects of vitamin D preparations since the interpretation to be placed on such assays so often depends on the statistical significance to be attached to them. The approximation of these distributions to normal curves has a further interest to other studies of physiological resistance, that is, the effect of inheritance on disease resistance, since it is often necessary to determine the perimeters of the curves for the resistance of the host to the given disease-provoking entity before an analysis of the inheritance of disease resistance is possible.

Evidence for the Effects of Inheritance on Resistance to Vitamin D Deficiencies

The mean durations of life of the different lines of rats, together with their standard deviations are given in table 5.

The data show several significant differences in the average duration of life between the lines. Such differences may be appreciated best by comparing the bar diagrams in figure 3. The height of the bars represents the average duration of life of the line indicated by number at the base of the bar. The left-hand portion of the bar represents the males; the right-hand portion the females. Evidently lines 6, 8 and 13 survived for the longest period on the vitamin D deficient diet. Lines 1 and 10 lived a shorter time, while the remaining lines showed the poorest survival.

The data show that the two sexes within a given line correspond fairly well in their duration of life. It is likewise evident that the lines which tend to live the longest time are those which show the differences between the sexes.

The relative effect of inheritance may be more clearly demonstrated by a rather precise comparison. The central idea behind inheritance is that closely related individuals, on the average, will resemble each

other more than will unrelated ones. The degree of resemblance between any two relatives may thus be determined as the quantitative

TABLE 5

Means and Standard Deviations of the Durations of Life of the Different Inbred Lines of Rats

Line	Mean		Standard Deviation	
	Males	Females	Males	Females
1	233± 9	210± 7	54± 6	56±5
6	289±18	258±10	90±13	37±7
7	133± 7	164±11	34± 5	55±8
8	295±14	221± 9	82±10	46±7
10	217±11	227±11	54± 8	50±5
11	132± 6	132± 5	33± 4	35±4
12	160± 5	158± 6	21± 3	40±4
13	344±15	274± 9	78±10	49±6
14	160± 7	151± 5	45± 5	36±4
15	144± 6	155± 9	43± 4	44±6
16	143± 7	116± 6	45± 5	40±4

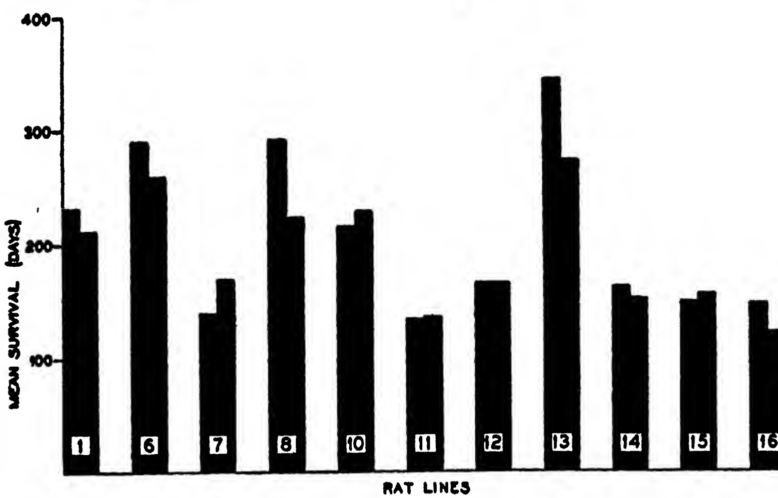

FIGURE 3.—Average duration of life of genetically differentiated lines of rats fed on a diet deficient in vitamin D. Left-hand side of bars, males, right-hand side, females.

difference between the measurements of a particular character in the two individuals. Genetically unrelated animals composing another

group may be treated in a similar manner. The difference between measurements for the related and unrelated or random sample groups will give a quantitative value for the effect of the inheritance. If now, we desire a direct measurement of the amount of genetic correlation between the related individuals in the groups the summed squares of these differences will give such a measure of this inheritance effect.

The total squared differences for the durations of life of the whole population of rats on this vitamin D deficient diet may be divided into two parts; the variation due to differences between the different genetic lines and the variation due to the random variation of the individuals within the lines. Since the genetic differences between the lines are the only known causative agents of variation, this variation may properly be attributed to the influence which the inheritance exerts on the utilization of any available vitamin D and of other elements in the deficient diet. The variation within each line may, for the moment, be considered to be induced by unknown environmental factors. The contribution of these two sets of factors to the total variability is seen below.

Males

Variation in whole population	1,334,400 days
Between related individuals	848,320 days
Within related lines	486,080 days

Females

Variation in whole population	690,720 days
Between related individuals	377,120 days
Within related lines	313,600 days

These results show that over half of the sum squares of the durations of life within the whole population are due to the differences between the genetically different lines of rats. For the males the contribution made to the whole variation by these racial differences amounts to 63 percent of the total. For the females the contribution is 55 percent of the total. The statistical significance of these differences may be determined in the usual way by comparing the variance of the between group class with that of the within group class, where the degrees of freedom are 10 for between lines and 159, males, and

156, females, for within the lines. These variances for the males are, between lines 84,832 days; and within lines 3,057 days; and for the females, between lines 37,712 days; and within lines 2,010 days. The variance due to the genetic differences is at least 18 times that within the racial lines where a difference of only 2.5 times would be significant. Both sexes thus agree in showing a well marked difference in the effects of the vitamin D deficient diet in populations of closely related individuals as contrasted with a group bred at random. The differences between the variances of the males and females, while fairly large could easily be due to random sampling since to be significant one difference would need to be more than 5 times the other.

There are three classes of genetic relationship for the individuals within the different racial lines. The first is that between individuals of the same litter; the second that between individuals of the same parents but different litters; the third that between the offspring of one pair with those of another pair of the same line. The variance contributed to the total by these different groupings of the data is found below.

These results are concordant in showing the closely related individuals to be quite similar in their duration of life whether they are born into the same litter, different litters of the same parentage or born of different parents but of the same racial inbred line. The results for the males vary somewhat, those for the females are rather constant, seeming to represent a more nearly average result. The variance within the matings—2,200 days for the males and 1,672 days for the females—is a fair estimation of the random variance unaccounted for by the differences in the racial lines. As pointed out earlier, the ratio between this variance and that between the racial groups shows that statistically speaking the odds in favor of an inherited effect on a dietary exhaustion are large indeed. Environmental effects common to the progeny of different parents within racial lines or to litters of the same parents also contribute elements tending to make their duration of life more nearly alike. If the variance of these two groups be compared with that of the within litters group, we find that to be considered significant, the former value should be almost twice the latter. Three out of four groups meet this requirement. The variance of one of the three groups, between

litters of the same parents in the males, is nearly three times that of the within litters group. These differences may be considered as indicative of some effect of common environmental or genetic factors (uncontrolled by the previous inbreeding) which were common to these particular groups. The net conclusion to be drawn from the evidence is, however, clear; the life-span of rats confined to the deficient diet is influenced markedly by genetic constitution.

	Degrees of Freedom	Sum of Squares in Days	Variance in Days
Males			
Whole population	169	1,334,400	
Between racial lines	10	848,320	84,832
Between different parents but within racial lines	19	82,400	4,320
Between litters of same parents	23	147,120	6,400
Within litters	117	256,560	2,200
Females			
Whole population	166	690,720	
Between racial lines	10	377,120	37,712
Between different parents but within racial lines	19	65,200	3,432
Between litters of same parents	17	47,760	2,808
Within litters	120	200,640	1,672

The Interrelation of Characters Affecting the Life-Span

It is of interest to inquire further into the inheritance effect and to determine, if possible, what characters the inheritance may affect and thus account for the observed differences between the genetic lines. Two such characters which vary with the different inbred lines are known to be present—the size of litter and the ability to reach a given initial weight at 46 days, the age when the deficient diet was commenced. These variables are interrelated. Both could conceivably be responsible for the observed inherited effects if they are found to be correlated with the survival times of the different lines. Furthermore it seems likely that these variables, litter size and weight at 46 days, would themselves be correlated. The correlations are presented below.

Besides the effect on the life-span, four other measures have been

used in estimating the effects of the defective diet, maximum weight attained while on the defective diet, weight just prior to death, days between start of diet and maximum weight, and days between maximum weight and death. The interrelations of these variables, as well as their relation to initial weight and litter size, have a direct bearing on the problem in hand.

Examination of table 6 reveals a close correlation between many of the variables. Such variables as age at maximum weight, maximum

TABLE 6

Correlations of Survival Time and Other Indicated Variables for Rats on a Vitamin D Deficient Diet

Characters Correlated	Correlated Coefficients	
	Males	Females
Weight at 46 days and age at maximum weight	0.43±.04	0.29±.05
Weight at 46 days and maximum weight	.82±.02	83±.02
Weight at 46 days and age at death	.60± 03	.50±.04
Weight at 46 days and last weight	.76±.02	.84± 02
Weight at 46 days and litter size	−.48±.04	−.46±.04
Age at maximum weight and maximum weight	.71±.03	.52± 04
Age at maximum weight and age at death	.85± 01	.76±.02
Age at maximum weight and last weight	.64±.03	.47±.04
Age at maximum weight and litter size	−.23±.05	−.06±.05
Maximum weight and age at death	.78±.02	.68± 03
Maximum weight and last weight	.91±.01	.91±.01
Maximum weight and litter size	−.39±.05	−.28±.05
Age at death and last weight	.67±.03	.58±.03
Age at death and litter size	−.34±.05	−.15±.05
Last weight and litter size	−.33±.05	−.38±.05

weight, last weight, are closely correlated with age at death, the coefficients being quite high, 0.85, 0.78, 0.67, for the males. These variables may be utilized to measure the defects of the diet and they also completely determine such other variables as gain or loss in weight on the deficient diet. This fact makes it entirely proper to confine our consideration of the inheritance to its effects on the life-span, since such other items are in large part only different measures of the same variable. The litter size and individual weight attained at 46 days, the time when the deficient diet was commenced, are in a some-

what different category in so far as their relation to the effects of the diet on the life-span is concerned. Weight at 46 days is rather highly correlated with the after life-span when the rat is on the deficient diet. The ability to reach a given weight at a given time is also an inherited characteristic. The correlations of table 6 suggest that the inheritance of this character is a contributing cause of the observed inheritance of the length of the life-span between the inbred lines. A similar observation may be made for the litter size. This latter case is somewhat different, however, since litter size may be a variable which expresses its effect entirely through its influence on the weight at 46 days. If this be true, all the attention may be given to the weight at 46 days. The partial correlation coefficients throw some light on the problem. The first order partial correlation coefficient between the weight at 46 days and the life-span where account is taken of the effect of the litter size is $0.53 \pm .04$ for the males, and $0.49 \pm .04$ for the females. The partial correlation coefficient for litter size and the life-span where the effect of the 46-day weight is properly accounted for is $-0.06 \pm .05$ for the males and 0.11 ± 0.5 for the females, correlations which are not statistically significant. The characteristic which accounts for all of the effect of litter size and also contributes something to determining the life-span under the conditions of the experiments is the weight at 46 days. The contribution which the inheritance of this character makes to the length of survival may now be investigated.

On the Character Basis for the Genetic Differentiation Shown in the Duration of the Life-Span

The inbred strains of rats utilized for these experiments differ markedly in their weights at 46 days of age, the period including nursing, weaning and growth. The broad ration which was used has maintained a colony of many rats throughout life and over a period of 15 years. Of the total squared sums of these weights for the whole population of males, 45 percent is contributed by the line differences. For the females the contribution is 57 percent. These contributions are statistically significant since the variance between the lines is for the males 13 times and for the females 19 times those of the variances within the lines.

The question of how much this inheritance is contributory to the inheritance of the duration of life under the unfavorable diet may be analyzed as follows. The sum of the squared differences for the durations of life of all rats was earlier split into two parts; that which is due to the differences between the inbred lines and that found within the lines. The question to be answered is, how much of each of these variations is due to the variations in initial weight? The numerical values necessary to answer this question may be determined from the correlation coefficients between the weights at 46 days and the life-spans within the two groups, between the inbred lines and within these lines.

These correlation coefficients are equal to 0.84 for the males and 0.62 for the females in the group where the variation is dependent on genetic differences between the inbred lines. Statistically speaking, they are significant. They are not significantly different from each other, however, since the difference is only 0.22 with a probable error of about 0.28. An average value of 0.73 for the correlation coefficient would thus be a fair estimate of the effect of the inheritance of weight on the life-span. The correlation coefficients between weight and duration of life within the inbred lines is the same for both males and females, 0.34. This is also significant since a correlation of only 0.21 would be exceeded by chance but once in 100 trials. This correlation of 0.34 within the inbred lines would seem to be due to two possible kinds of variables; inheritance heterozygosis, which is as yet uncontrolled by the inbreeding, and common environmental factors having common effects on growth and life-span. It is significant to note that correlations due to such causes are only half those due to the controlled genetic causes, showing that to this extent, at least, the inbreeding has segregated important hereditary factors for life-span and weight into fairly pure racial groups.

The amount of the variation in life-span due to differences in the inheritance for weight may be determined from the relation

$$v = V(1 - r^2)$$

when V is the variance of the life-span, r is the correlation coefficient between weight at 46 days and the survival time and v the partial variance, that is, the variance remaining after account has been taken

of the effect of the inherited variation in weight. By substituting the correlation coefficient of 0.73 in this equation we find that the variance remaining after account is taken of the effect of the weight at 46 days is only 47 percent of that where weight varies as it will. Of that portion of the total variance attributable to inherited differences between the rat strains (59 percent), 53 percent or 31 percent of the total is contributed by inherited differences in weight. The remaining 47 percent of the inheritance effect on duration of life is due to characters which are at present unknown. The same reasoning may be applied to the variation in length of life within the different lines. The variation due to the uncontrolled inheritance of weight within the inbred lines or to common environmental factors is measured by a correlation of 0.34. The variance remaining after proper account is taken of these factors is 88 percent. The portion of the variance within rat strains due to this character, weight, is consequently but 12 percent of that observed within the rat strains. The tabulation of these results is as follows. The total sum of the squares is reduced to a percentage, 100, and the contribution of each of the different factors to this variation listed as a percentage of this total.

Total variation in life span (sum of squares)	100
Variation between genetically different lines	
Percent of variation due to inheritance of weight	31
Percent of variation due to unknown characters	28
Total attributable to inheritance factors	59
Variation within genetically similar lines	
Percent of variation due to weight	5
Percent of variation due to unknown environmental characters	36
Total attributable to chance inherited and environmental influences	41

In general, the evidence leads to the following conclusions. Since the only known variable which tends to differentiate the inbred lines is heredity, the evidence indicates that somewhat more than half (about 59 percent) of the variation in life-span under conditions made unfavorable by a deficiency of vitamin D in the diet, is due to characters under hereditary control. Approximately 36 percent of the variation is due to unknown environmental influences. Of the half due to heredity, a half of that, or a quarter of the whole, is attribut-

able to the inheritance of the character weight at 46 days. The other quarter, although definitely due to heredity, must be attributable to the inheritance of characters unknown but important to survival under the prescribed dietary conditions. Only 5 percent of the variation within the lines is assignable to weight variations and 36 percent to unknown but nevertheless very real environmental or uncontrolled hereditary factors. The data from which these comparisons are drawn are given in table 7.

TABLE 7

Analysis of the Variation of Weight at 46 Days and Life-Span in Strains in Inbred Rats

Origin of Variation	*Degrees of Freedom*	Weight at 46 Days (Gms.)		Life-Span (Days)		Correlation	
		Sum of Squares	Variance	Sum of Squares	Variance	Cross Products	Correlation Coefficient
Male							
Whole population	169	58,010	334 3	1,334,400	7,896	166,630	0.599
Between strains	10	26,073	2,607.3	848,320	84,832	124,210	.835
Within strains	159	31,938	200.9	486,080	3,057	42,420	.341
Females							
Whole population	166	37,785	227 5	690,720	4,161	80,710	0.499
Between strains	10	21,655	2,165.5	377,120	37,712	56,350	.624
Within strains	156	16,130	103.5	313,600	2,010	24,360	.343

Some General Biological Aspects of the Data

The data favor the view that within a species the reactions of individuals to apparently essential dietary constituents may differ and that this variation is controlled partly by the inheritance. It is of interest to digress and consider here the consequence of such variation in evolution. In the shifting of species from one locality to another of quite different dietary possibilities, it would seem that extensive modification in the physiological economy of the group would often be required. Such, for instance, would be the case of groups moving from high, dry plateaux over mountains into low, hot, moist regions; or, in the laboratory, in the transfer of bacteria from a susceptible host to culture media and *vice versa*.

Two explanations for such modifications in evolution seem possible. First the group could move into the unfavorable area and through some action of the environment become modified and survive the conditions imposed. These modifications, of course, would have to be hereditary and the objections can be raised that the germ plasm is not readily altered and that only a small proportion of such modifications as do occur improve the survival value of the animal. The chance of establishing the initial foothold in the unfavorable area would thus be slight. An alternative proposition would be that within a group certain animals may become potentially suited in their genetic constitutions to environments different from those in which they are found, but into which they can migrate. These extreme forms which would rise at random and without an yevident adaptive relation to the environment would thus be fitted to meet the new environmental conditions and to establish themselves in sufficient numbers to gain a foothold. Further mutation and selection, even though the rate be small and the effect slight, would eventually tend to complete the adaptive change.

The évidence obtained in our experiments points to distinct genetic differences in the dietary requirements of strains of rats. These differences existed before there was any known exposure of the race to dietary deficiencies. Certain lines were adjusted by their previous inheritance to a somewhat lower requirement of given food elements than were others, and could, consequently, meet the lack of such elements more successfully. The fitting of certain members of the group to the environment came first rather than as a consequence of previous modification.

This view is supported in the experiments on disease resistance, in which the different inbred lines of a race are exposed to a disease rare to it. Examples include pseudorabies of cattle introduced into mice (GOWEN and SCHOTT 1933) or *B. abortus* of swine in rabbits (COLE 1930), since in both instances it has been possible to demonstrate that certain strains have inherited resistance to the disease, whereas others show distinct susceptibility to it.

Other diseases which are known to be quite dependent upon the genetic constitution of the host for their infectivity do not furnish such critical evidence, since these diseases occur commonly in the

species and selection for susceptibility or resistance may have occurred.

Such diseases as rat typhoid, *S. enteritidis* of IRWIN (1929), mouse typhoid *S. aertrycke* of SCHOTT (1932), *B. enteritidis* of WEBSTER (1933), white diarrhea, *S. pullorum* of ROBERTS and CARD (1926), typhoid, *S. gallinarum* of LAMBERT (1932) fall into this group, since it is uncertain whether the fitting of specific groups to the environment came first or as a consequence of previous modification.

The bacteria and especially the protozoa furnish significant evidence of both types of genetic modification of the host organization. Evaluation of the results necessitates a slight reorientation in thought, since the selection may affect the soma instead of the germ plasm in these rapidly reproducing unicellular forms. In certain bacteria, the colon group, for instance, division may occur every 20 minutes. A single organism then, in the course of 8 hours could give rise to 16,000,000 individuals if all survived during the period. Since the possible rate of mutation for a specific gene is 1 to 10 in 10,000,000 individuals nearly every gene and the character or characters which it governs could have changed at least once in 24 generations.

Wild species of paramecium (JENNINGS 1908), difflugia (JENNINGS 1916, 1929), centropyxis (ROOT 1918), yeast (HANSON and others 1906), colon bacillus (BARBER 1907), and many others have shown several biotypes of diverse heredity, each of which is capable of reacting differently to the same or to diverse environments. Experimentally, the environmental agents used were in some cases poisons with which the organism presumably had had no previous contact,—the parasitic trypanosomes (TALIAFERRO 1926) or the free-living paramecia (JOLLOS 1921).

The choice of the original biotype seemed fortunate in certain cases (JENNINGS 1929) since the results were not always reproducible with other stocks. The effects followed a pattern closely similar to that observed in other less prescribed environments, in which for example the criterion was that the animals should reach a given size or survive a certain heat. The initial contrast between the selected lines seemed to be due to previously differentiated biotypes selected from the wild population. Further selection and presumably further purification of these biotypes resulted in further progress, that is, increase

in size or resistance to the heat or poison. If still further progress was attained it occurred in more or less discrete steps of unequal length in both generation time and advance toward the greatest adaptibility for the environment. This goal, as DALLINGER (1887) showed, may be far removed from the ordinary environmental conditions under which the species are accustomed to live: in 7 years time the temperature requirements of flagellates were changed from 60°F. to 158°F., 16°C. to 70°C. But these changes in the genetic structure of the organisms are no more permanent than those found in the wild type from which they started many generations earlier. For, if the environment is now changed, the forms may again, by a slow step-like process, be made to return to forms whose requirements are those of the wild stock from which it originated. This result is not so surprising if cognizance is taken of the relatively large numbers of animals involved, and the rates at which genes vary under natural conditions. It suggests that in any population, as in the rat population here studied, variation in the nutritive requirements, etc., are occurring which are purely outside the requirements of the given environment and that if the environment should change those variants having the capacity to meet the changed conditions become the parents of those which are to form the new race.

SUMMARY

The data presented here show that rats which are fed a low vitamin D, high calcium diet, from the 46th day of age have an average duration of life of 5 to 6 months, or not quite half, that of like animals on a normal diet. Rats on the deficient diet die off more sharply than those fed normally. The standard deviations of duration of life of rats on the deficient diet are less than half those of animals on the normal diet. The relative variation is consequently greater in the normal rats. It is further noted that males on the vitamin D deficient diet live slightly longer than the females, but on a normal diet the results are reversed. The frequency curves of death show a distinct skewness.

Data for the separate lines show distinct differences between these lines in respect to length of life. The variations range from 131 to 345 days for the males and 132 to 267 days for the females. A com-

parison of the variations found for the whole random bred population and for the rats related by strain, shows that a distinct correlation exists between the duration of life of the animal within a given line. The correlation coefficient for the males is 0.63; that for the females 0.55.

Within the strains there are 3 relationships which bear on the strain difference: correlations between members of the same litter, between individuals of two litters and the same parents, and between individuals of the same line but of different parents. The analyses of the effects of these relationships are concordant in showing that the genetic differences between the racially differentiated lines account for the greater part of the variation. Variation due to the similarity in environment of offspring of the same parents or of the same litter are rather small and statistically just significant.

The hereditary differences between the inbred strains are found to account for somewhat more than half of the variations observed in the life-span. Of this hereditable variation, about half seems to be due to the inheritance of the character, body weight at 46 days. The remaining quarter of the variation in life-span due to heredity may be attributed to the inheritance of characters as yet unknown but important to survival under the dietary conditions imposed by the experiment. Of the variation remaining after account is taken of the inheritance effect, only 5 percent is attributable to variation in weight. The other 36 percent is due to unknown factors apparently largely of environmental origin, although even here some of this variation could be due to some heterozygosis of the germ plasm as yet uncontrolled by the inbreeding.

Finally, the evidence considered from a broader biological viewpoint has a bearing on the problems of adaptive evolution which may be significant. The suggestion is made that the fitting of certain groups within a species to particular environmental conditions would seem to come first rather than as a consequence of modification by the environment. The experiment presented above supports such a contention, for the evidence points to distinct genetic differences in the dietary requirements of the strains which, so far as it is known, never had been exposed to the particular deficiencies involved.

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TAPEWORM STUDIES

II. PERSISTENCE OF THE PASTURE STAGE OF M. EXPANSA

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In addition to apparent lack of ability in its pasture stage to spread easily if at all (Stoll, 1935), a second striking epidemiologic attribute of Moniezia is its persistence in an already infested field. Survival of the infective stage over periods when sheep are not present, or if present are not harboring the cestode, is well demonstrated in our observations. The fact is of biological interest as well as basic in projecting control and eradication measures against this tapeworm. It may be assumed that similar conditions obtain for members of the Anoplocephalidae other than those of ruminants.

Of the two Moniezia species in our flock, the predominating form is *M. expansa,* and the present analysis deals with it, we believe exclusively. The second species manifests apparently similar epidemiology, in some respects somewhat less clearly defined. Exposition of its characteristics is reserved for a later article.

The pasture involved is field I (see map, Stoll, 1935) of 4.8 acres. On March 1, 1931, field II of 3.5 acres became an intercommunicating pasture with field I, and has so continued. The sheep population was, of course, a changing one. Its census shows that seasonal stocking varied from 1¼ (1926) to 4 (1930–31) animals per acre, the monthly average 1926–32 being about 3.

Observations are considered in chronologic order, beginning with an early period by Smith and Ring (1927), whose flock came under the author's study in the spring of 1927.

In figure 1 the data of 1926–32 are presented graphically. Oblongs in the chart show, by their position in relation to the calendar scale, when animals were introduced and removed from the pasture. *M.*

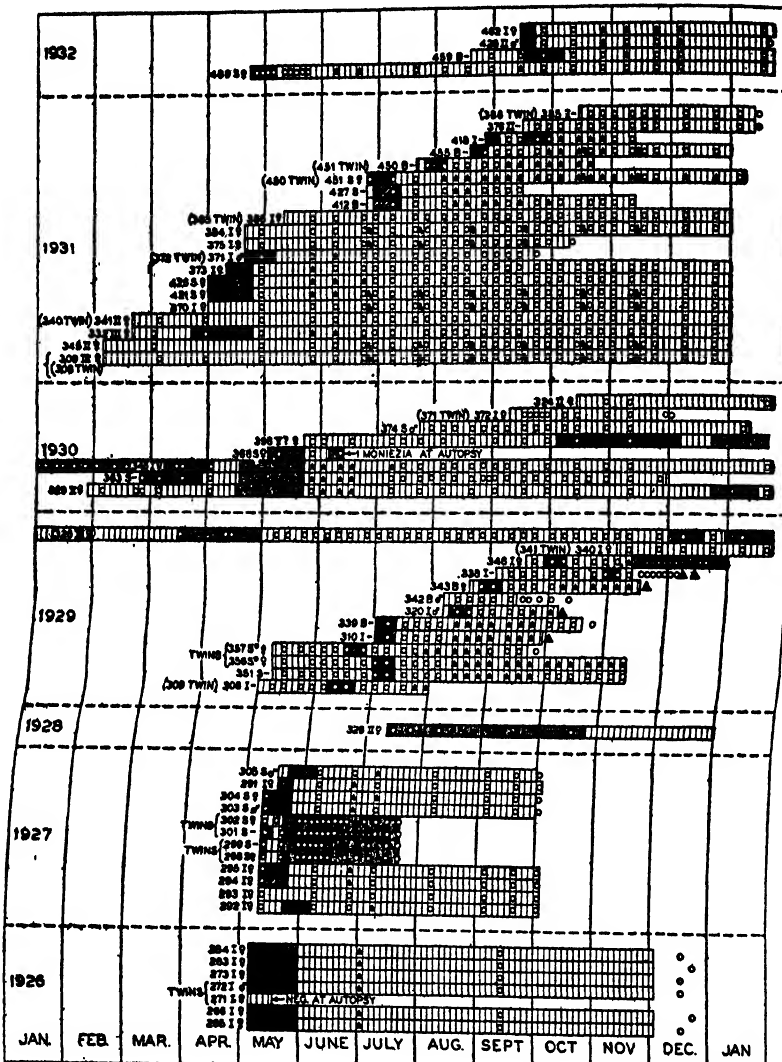

FIG. 1. A synoptic presentation of the data discussed in the text.

Unstippled oblongs show when the sheep were in the infested pasture, stippled portions when they were out of it. (Stippling is omitted where no confusion results.) Broken ends of oblongs indicate continuance. Following the animal numbers, B stands for a lamb less than ½ year of age and bottle raised on cow's milk, S for suckling lamb (twins no. 356 and 357 were born on pasture), I, II, a yearling, two-year-old, etc. The sex symbols for castrated males (wethers) are omitted, and a short dash used instead. Triangles represent fecal examinations

expansa positives (by flotation) are marked by triangles, which represent the usual microscopic appearance of these tapeworm eggs. For simplification, examinations preceding introduction of the animals to pasture are omitted, all having been negative for tapeworm.

Animals up to 26 weeks of age are considered lambs, 27–78 weeks as yearlings, 79–130 weeks as two year olds, etc.

Our determination places the beginning of the patent period of *M. expansa* in sheep at 35–38 days, and perhaps as early as 34 days after infection. (Seddon, 1931 c, using an unstated method of fecal examination, found grazing lambs in Australia positive at 40 to 42 days after last preceding negatives at 35 to 37 days.) With this information it has been possible to add to figure 1 the indication of when each particular sheep became infected on the pasture. Shaded areas, which show this in the oblongs, are to be considered conservative determinations. They are calculated from infection having taken place 34 days before the initial Moniezia-positive fecal examination, to infection 38 days antecedent to the day after the last negative which preceded the initial positive. In this way all such probabilities appear to be covered, as hosts actually positive the first time on the day of the initial positive examination, to those having become positive on the day after the last preceding negative. Figure 1 represents these determinations to the 3-day periods of the month in which the calculated dates occur. Obviously for any animal, the longer the shaded portion of the oblong, the less precisely defined is the period when infection was contracted, it being dependent not only on the number of days between fecal examinations, but on the relation of such examinations to the date of entering infestive pasture.

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positive for *M. expansa*, circles negative. For animals off pasture the triangles are left open to call attention to the fact that such infection was not contaminating the pasture. The rule has been followed where possible of giving the examination record to a point where the *M. expansa* state of the animal leaving pasture or at the end of the year is defined. On the calendar scale the monthly intervals are divided into tenths, the first nine of which represent 3 days each, the last the remaining 1–4 days of the particular month. As the text explains, the periods within which the known positive animals contracted their infections have been calculated, and are shown by shading of the oblongs. The more precise such determination, the shorter the oblong. In 1931 several animals were treated, shown by ℞. Copper sulphate was used except for no. 455 which received tetrachlorethylene.

It is to be emphasized that without exception the data include all susceptible sheep introduced to field I. This covers all that were raised at the laboratory and three purchased ewes, no. 328, 329 and 396, whose susceptibility was shown by their contracting infection. Animals once having been infected characteristically do not show *M. expansa* again, as suspected by Morgan (1925) and demonstrated by Seddon (1931 b). Furthermore, animals in which infection was not observed their first exposure season have not later given evidence of it, as judged by consistently negative fecal and autopsy examinations. Similar negatives have been the rule with the occasional ewes purchased for their oncoming lamb crop. Such ewes have been secured primarily from two local farms, upon each of which *M. expansa* is known to be present, and the later failure of such animals to show the tapeworm may be accepted as indicating past infection when young. All told, in the period ending 1932, there were 78 sheep insusceptible in the infestive pasture as evidenced by more than two thousand examinations, all negative for *M. expansa*. These include some of the 63 animals, whose approximately one thousand examination records as susceptibles are given in figure 1, during their later periods of one to five seasons in field I. They will come under review in more detail in connection with a later article. Five goats, bottle-raised, and in field I at times from 1930–32, showed no sign of tapeworms.

OBSERVATIONS

Persistence of Infestation in Unused Pasture

A record of *Moniezia expansa* viability on fallow pasture for nearly a year and a half has already been referred to by Smith and Ring (1927). The instance deserves more detailed record, not only because it represents the longest pasture survival so far reported for this parasite, but because certain cognate facts render the observation a convincing and significant one. In addition it forms chronologically the natural introduction to our own series.

Field I, first fenced and seeded for grazing in 1922, received from paddock A on May 10, 1923, a flock of 26 adult sheep and 22 spring lambs, another being born in June. *M. expansa* appeared in the flock during the summer, probably brought in by the purchased breeding ewes which also harbored several nematode species. The fact that

23 lambs were in this group through 4 months suggests that field I was being seeded with numerous Moniezia eggs. On September 12 the sheep were driven to paddock A. Lamb no. 209 killed November 8 had a heavy Moniezia infection.

Part of field I is swampy. The balance, denoted Sassafras loam and a good soil for grass, was again ploughed and seeded in the fall of 1923. In the spring of 1924 the flock, then consisting of 23 adults and 13 spring lambs, was driven from paddock A back to field I. On September 12, 1924 lamb no. 253 died, showing at autopsy not only numerous Haemonchus but about 30 tapeworms. The pasture again evidently received many Moniezia eggs from this lamb and from some or all of the other dozen.

On November 25, 1924 the flock was removed from field I, which was not again in use for 17⅓ months. During this interval it was entered only in December 1925 when the grass was burned from much of the field. Good pasturage resulted the following spring. In 1925 Smith and Ring had raised on cow's milk a group of lambs which had been kept since May 5, 1925 in enclosures on uninfested fields. Seven of these, none having shown evidence of helminths, were placed in field I by themselves on May 5, 1926. Ewe no. 271 was killed May 17 because of an everted uterus. She had been on pasture 11 days and showed no parasites at autopsy. The remaining six yearlings were all positive for *M. expansa* on fecal examination July 1.

Since this examination did not occur until nearly 2 months after the animals had been placed in pasture, the time during which the tapeworm was contracted cannot be narrowed beyond the 23 days, May 5–28. It is nevertheless noteworthy that the tapeworm was ingested by every one of the remaining six animals not later than May 28. A chance fact probably permitted this rather prompt contraction of Moniezia on a field ungrazed so long. The burning of the dry grass the preceding December, with the subsequent roughening and washing of the soil surface through winter effects, did not disturb a good grass growth in the spring. It must, however, have enhanced any opportunity for organisms present in the upper layers of the soil to be obtained by sheep which were on new pasture after living partly on hay in an enclosure in field IV, and consequently now grazed with greediness.

Such evidence of persistence of the infestation in field I is of special

interest, covering as it does a span of two winters. This period of $17\frac{1}{3}$ months is to be regarded as the minimum determination, since it assumes deposition of tapeworm eggs on the pasture right up to the time the flock was removed from it in November 1924. While field I was the lowest portion of the drainage basin upon which sheep had been pastured, there was no active tapeworm infection recognized in the summer of 1925 in the only other group of sheep on the place in field V, the flock in paddock A on another drainage slope having been sold in April and May 1925. Even if there had been such infection, it would have needed to reach field I from field V in the waters of the brook which traverses field I, presumably in May 1926 itself. Such a fortuitous grouping of events seems distinctly unlikely, especially when this instance of survival on field I fits into the picture supplied by the additional instances of viability noted below.

An added observation may be given emphasis. This particular group of sheep which contracted the tapeworm in field I in May 1926, occupied in 1925 an enclosure about 100 feet away in an orchard just across the road from field I but uphill from it. The failure of Moniezia to move that short distance in the 8 weeks July 8 to September 11, 1925 is the more striking when it is in mind that the pasture itself was at the same time unoccupied, and these very animals demonstrated its infectivity 8 months later.

Field I furnished a second demonstration of persistence over the winter of 1926–27. The Smith and Ring sheep when examined in September 1926 did not show Moniezia, nor did any of them ever do so subsequently. They were removed from pasture on December 2, 1926, no more animals being in field I until May 9, 1927. At that time, 8 months after the negative examinations of the preceding September and after the pasture had been out of use for over 5 months, *M. expansa* promptly developed in four of five yearlings, and five of seven lambs.

The infections contracted in May 1927 (fig. 1) are marked by the evident promptness with which two of four lambs in the pasture secured the tapeworm, while the other two escaped it in an 11-day exposure. Sheep can show *M. expansa* with an infection of even one tapeworm, as Smith and Ring (1927) found for their ram 290. Our records include other examples. Perhaps these 1927 infections mean only one or a few tapeworms for each animal, and the pasture survival

here was merely a light or moderate infestation. In the absence of autopsies this was not determined. One ewe, no. 293, failed to exhibit the infection. Conceivably the particular examination schedule missed her positive phase. However, this ewe may have become immune following an infection missed in November 1926 when ram no. 290 was positive, all these animals then having been in the same enclosure.

It is of interest that if the 1927 sheep had been examined only on July 1, as in 1926, all known positives would apparently have been demonstrated, and as is clear from figure 1 an infection picture almost identical with 1926 have resulted.

It is accepted that field I produced still a third demonstration of long persistence of Moniezia in its pasture stage. Of the sheep noted above which were infected in the spring of 1927, some showed their final positive June 27, others July 8, all being Moniezia-negative August 8. The last of these animals were removed to paddock C on October 3, and the pasture went unoccupied nearly 13 months. In late October 1928 the following animals were placed in field I:

(*a*) Three which had shown no evidence of tapeworm for 15 months on 159 examinations. This accorded with expectation as ram no. 272 had had *M. expansa* in 1926, and ewes no. 291 and 304 in June 1927.

(*b*) Dorset ewes no. 327 and 328, secured from Ithaca, New York, in early July 1928. Number 327 was then showing *M. expansa.* Both were negative July 24, Aug. 9 and Oct. 16, after which their stay indoors was terminated by transfer to field I.

(*c*) A 7 months ewe, no. 334, which as a pet had been tethered on the lawn of its owner in a nearby village throughout the summer. No evidence of tapeworm had been observed, although no microscopic examinations had been made. Endemicity of *M. expansa* in the locality makes it likely that this animal had had the infection as a young lamb.

(*d*) Fourteen bred ewes, none less than 5 years old and none of Dorset blood. These were not examined at the time, but animals from the same farm before and since had indicated Moniezia infestation there, so that it is accepted that all had had tapeworms when young animals, and were neither infected nor susceptible to *M. expansa* when purchased.

None of these twenty sheep received fecal examinations during the

next two months, November and December. Between early January and early April all of the fourteen old ewes and the ram came to autopsy, some after extensive examinations covering 2 months. No sign of cestodes was observed in any of these at any time. In January, ewe no. 334, and in March and April the remaining four adults began getting practically weekly examinations which continued throughout the year. Ewes no. 291, 304, 327 and 334 showed the square eggs of the second Moniezia species at intervals between September and December 1929, and in January 1930 ewe no. 328 exhibited *M. expansa*, evidently contracted in December 1929. Infections of *M. expansa* which resulted after known susceptible animals were placed in this field in early May 1929 are thus considered to have come from infestation persisting on the pasture since July 1927, a period in excess of 22 months.

The lack of examinations in November and December 1928 prevents this from being a definitive determination, but probabilities strongly favor it. Present knowledge of Moniezia infection and of local conditions renders it unlikely, as already mentioned, that any of the animals placed in field I in October were infected at the time. The young ewe no. 334 was the particular animal likely, if any, to contract *M. expansa*. (While the Dorset no. 328 is later shown to have secured the tapeworm in December 1929, that very fact renders it improbable that she had had the infection earlier. In field I every season since, she failed to show *M. expansa* again until 1935.) If ewe no. 334 obtained the tapeworm in the fall of 1928 and seeded the pasture with eggs, it means she contracted it promptly under conditions when the infestation had already survived 13 months and when close cropping of grass in a long fallow field would not have been a likely happening. Both items, together with "insulation" of the sheep from the pasture "floor," through approaching winter conditions and feeding of hay, would suggest delay in contracting the infection. In addition supposedly developed mature stages of the tapeworm must in turn have been lost soon, for she was continuously negative indoors beginning 12 weeks after purchase.

The infections developed in the spring of 1929 have evidential value here. The first 1929 positive occurred on July 22 in yearling no. 308 which had been in field I since May 1. Calculation of the date of securing this infection places it between June 9 and 19, which

means that this wether grazed more then $5\frac{1}{2}$ weeks before ingesting the tapeworm. As figure 1 shows, three lambs present beginning May 7, showed longer lag periods. This intrinsic evidence is more consistent with the procuring of infestation that had persisted in field I for nearly 2 years than from a pasture reseeded with Moniezia in recent months.

It may be noted in figure 1 that altogether six animals introduced to field I in early May and early July 1929 contracted *M. expansa* before any new eggs were being deposited in the pasture. Subsequently five more animals are shown to have become infected, in August to December. One of these, Dorset ewe no. 328, after being in field I continuously for 13 months except for one brief interruption is shown to have become infected in December. This ewe lambed January 14, 1930. Oppositely two apparently susceptible animals, ram no. 342 and ewe no. 340 failed to exhibit the infection, although present in pasture during a period of known infectivity.

A fourth instance of persistence of the infestation in the absence of sheep was provided by a small enclosure in field II (labelled SP in fig. 1, Stoll, 1935). This area of 4500 sq. ft. is separated from field II proper by a 9-foot dead space. The slope of the land inside the plot and dead space is toward the pasture, not at all in the opposite direction. *M. expansa* eggs were deposited in the enclosure by infected lambs between September 5 and October 12, 1933, and perhaps somewhat later. On November 4 the last animal was removed and the plot contained no other sheep and was not entered until May 31, 1934 when a susceptible lamb was introduced. This lamb was shown positive for *M. expansa* 57 days later, having contracted the tapeworm between June 12 and 23. Its grazing area represented stocking at the rate of about ten animals per acre. Here the infestation survived for 7 months. Presumably this well-determined observation would have another meaning provided Moniezia infection was able to migrate against gravity (a condition contrary to fact in this laboratory; Stoll, 1935), for known infestive pasture was hard by this enclosure on two sides, beyond the 9-foot insulating grass strip.[1]

[1] The winter 1934–35 furnished an additional instance of 6 months persistence of the tapeworm in this enclosure. With *M. expansa* eggs being deposited at least until December 4, 1934, sheep were all removed a week later and the enclo-

Climatic Factors

The observations noted in regard to persistence of Moniezia infestation on pasture indicate that in this region it overwinters successfully, and may survive intervening seasons also. The determinations extending from the fall of 1924 to the summer of 1926 by Smith and Ring, and thence on to the summer of 1927, are of such simplicity and precision as to make it of value to record some of the climatic factors resisted well by the pasture stage of this parasite. Especial interest attaches to moisture conditions, as dryness has long been accepted as unfavorable to Moniezia. It so happens that these years possess the added interest of including several marked subnormal precipitation records. Persistence under such conditions implies equally good or better survival in periods of lesser dryness.

In figure 2 are shown mean temperatures, snowfall, and total precipitation by months from September 1924 to May 1927. The data are from the U. S. Weather Bureau at Trenton, 12 miles away.

Known deposition of *M. expansa* eggs was taking place in September 1924 and may have continued until the flock was driven from pasture in November. October had the exceptionally low rainfall of 0.2 inch, the driest month in the Trenton records, which go back in part to 1865. As a matter of fact, this 0.2 inch shower on October 8 was the only measurable precipitation in the 6 weeks between September 30 and November 14. Of interest also is the low rainfall of 0.7 inch in June (only one other June, that of 1913, is recorded for Trenton with so little rain) and 1.1 inches in August 1925, though precipitation in July at 5.1 inches was in excess of normal. The annual Weather Bureau summary states, "The two principal weather factors, temperature and precipitation, show that the year 1925 was warm and dry in New Jersey." But the degree of dryness locally was evidently unable to produce any marked teniacidal effect on the field infestation. A factor contributing to pasture survival of the tapeworm in the summer of 1925 may well have been the protection afforded against excessive drying by the extent of grass growth in a field then unoccupied.

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sure not subsequently used until June 17, 1935, when a susceptible lamb was introduced. This animal evidently ingested the tapeworm very promptly, passing eggs 36 days later.

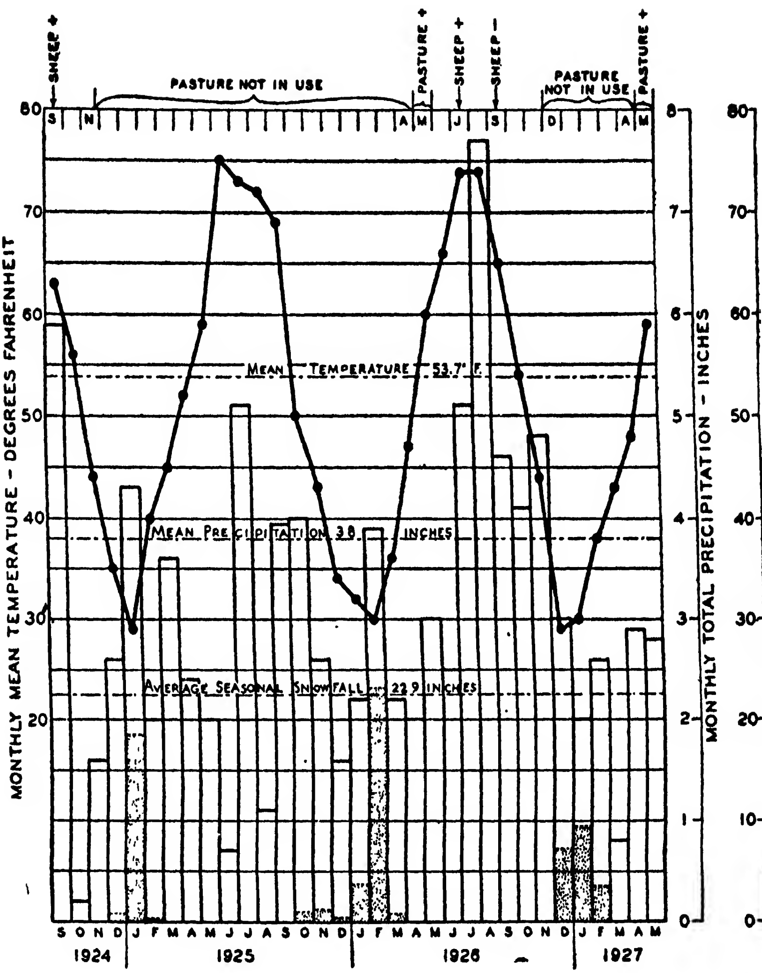

FIG. 2. Snowfall, total precipitation (which includes melted snow), and mean monthly temperatures from September 1924 to May 1927, in relation to two successive periods of *M. expansa* persistence on pasture of 17½ and 8 months respectively.

Data are from the U. S. Weather Bureau at Trenton, 12 miles away. (The record is extended through October 1927 in Stoll, 1932.) March 1927 with 0.8 inch rainfall is the driest March, and October 1924 with 0.2 inch is the driest of all months in the Trenton records, which go back, in part, to 1865. Monthly means of temperature and precipitation have this base also. Average seasonal snowfall covers 1912–13 to 1933–34 inclusive.

The rest of the period until May 1926, when susceptible sheep became infected, does not show striking variations from "normal" conditions, except for the unusually early snowfall in October 1925, which is another Trenton Weather Bureau record. Average monthly temperatures ranged characteristically from 29°F. in January 1925 to 75°F. in June.

During July 1926 Moniezia eggs were being deposited again on pasture in a period of good rainfall. No month of marked deficiency occurred until March 1927 with 0.8 inch, the driest March in the Trenton records; but this again imposed insufficient soil drying to eliminate the pasture infestation, for the susceptible sheep entering field I in May promptly contracted the tapeworm.

The 22 months from July 1927 to May 1929 had no rainfall lower than 1.5 inches in December 1927, and the period may be thought of as more favorable in that respect than the earlier ones noted.

Snowfall is in general light to moderate in this region, and usually melts away shortly so that the ground is bare most of the winter. Its blanketing effect does not appear therefore to be the prime factor in the generally successful overwintering of Moniezia, although the 18 inches of January 1925 and 23 inches of February 1926 may have been of protection to the tapeworm during the long survival period already discussed. The total seasonal snowfalls for ten successive winters beginning 1923–24 were respectively 28, 19, 30, 20, 19, 10, 9, 5, 5, and 23 inches. The following winter of 1933–34 was exceptional with 40 inches at Trenton and 55 inches at the laboratory, where 39½ inches fell in February 1934, a month when the mean temperature at 19°F. was 12° less than normal and reached a minimum of −14°F. This is the winter in which the small enclosure in field II showed persistence of the infestation. While laboratory tests indicate *M. expansa* eggs are resistant to low temperatures, it is not unlikely that the excessive snowfall locally of this particular season was of assistance in permitting survival of the pasture stage of Moniezia in this somewhat unfavorably situated enclosure.

SUPPLEMENTARY OBSERVATIONS

Tapeworm Persistence in Pasture with Sheep not Then Harboring M. expansa

Supplementary to the evidence of *M. expansa* survival during periods when an infested field is free of animals are instances of its persistence when sheep are present, but no longer harboring the tapeworm. This is the usual state of affairs with permanent pasture; lambs become infected in the spring, and after they lose their tapeworms later in the season, the pasture does not get more *M. expansa* eggs until the following year. The individual parasitic histories of our sheep are known in sufficient detail to permit the two following determinations, made when the pasture held the flock continuously.

In 1929 the last sheep passing *M. expansa* eggs was removed from field I on November 16. Of susceptible animals placed here the following January, February and March, it is shown that the infection earliest contracted was between February 24 and March 26, 1930, a relationship indicating a minimum survival period of the pasture infestation of $3\frac{1}{3}$ months. This was a winter of little snow, December, January and February having but 3 inches each.

In 1931 the last sheep passing *M. expansa* eggs was positive December 23 and negative January 5, 1932. A susceptible lamb placed on pasture April 22, 1932 contracted infection between then and May 4, a survival period of the pasture infestation of about 4 months. This again represents an overwintering in the absence of good snow coverage, total fall for the season being only 5 inches distributed over the 5 months November to March.

A possible additional instance of survival over the 1930–31 winter is later discussed.

Presumptive Case of Direct Infection

Epidemiologically 1930 proved an interesting year, for it furnished first, an instance—the significance of which was not grasped at the time—of infection with *M. expansa* apparently by recently deposited eggs in paddock A, and thus presumptive evidence of direct infection; and second, of upset infective conditions in field I during a period of drought.

Three animals, no. 329, 363 and 346, were placed in field I on January 28, February 24 and March 20, 1930 respectively. Ewe no. 346—one of our few demonstrable cases of reinfection—had exhibited *M. expansa* eggs in November and December 1929, and, negative on numerous examinations indoors during January, February and March, rather promptly again contracted infection in the latter part of March when replaced in pasture. Lamb no. 363, introduced into field I February 24, secured infection about the same time as ewe no. 346 or somewhat earlier. They definitely establish the presence of the overwintering pasture infestation as already mentioned.

On April 17 these three animals, with the rest of the flock, were transferred to paddock A for 5 weeks. On April 29 lamb no. 363 was passing *M. expansa* eggs, and ewe no. 346 similarly on May 7. Each may have been doing so several days earlier. Ewe no. 329, not examined then, was negative May 27 and positive June 3. Figure 1 illustrates these relationships clearly. Calculating the date of infection from first positive and last preceding negative examination, as explained earlier, places the infection of ewe no. 329 between April 20 and 30, during the time she was in paddock A. It is evident that, considering the time intervals involved, occasion would hardly have been afforded for the establishment of infection in any intermediate host, and direct infection by recently passed eggs from no. 346 or 363 seems indicated. The alternative is to grant an exceptional prepatent period to the infection of no. 329, or that she was irregularly positive (as, for instance, no. 412 in 1931, no. 428 and 489 in 1932) and conclude it was contracted during the last of her stay in pasture. Additional examinations of no. 329 would have made the decision clear. In their absence the alternative is conservatively assumed, and the infection of no. 329 ascribed to pasture I, as figure 1 shows. The incident is of special interest in view of the fact that no. 329 had resided in paddock A for a year and a half without tapeworms before going to field I on January 28.

M. expansa Epidemiology in a Period of Drought

Following evacuation of field I on April 17, 1930, this pasture was ploughed and reseeded. While the grass got a fair start before the sheep were returned to it on May 21, the season turned out to be

abnormally dry. To quote the 1930 Weather Bureau Summary for New Jersey: "Except for the interruption during June, the condition was one of persistent dryness and almost as continuous warmth. From the middle of July to the middle of August, the deleterious effects of the dry weather were increased by high temperatures. August: Some let-up in the heat wave, but the drought continued. September: The average temperature was the third highest on record for September. Drought continued. October: Continued dry. November: Nearly normal conditions. December: Drought conditions resumed." These conditions carried over into 1931: For New Jersey "the year was the warmest on record (47 years) and the second driest. The rainfall—only slightly more than that of the drought year of 1930—fortunately, was rather concentrated in that important part of the growing season from March to August inclusive."

From April 1930 until May 1931, the average Trenton rainfall was 2.2 inches per month, about 58 per cent of normal, the driest month being September 1930 with 1.2 inches.

This set of weather conditions, following the plowing of field I which undoubtedly buried much old infestation, is related probably in a causal way to the Moniezia events on pasture for about 12 months. Unwittingly, it produced almost an experimental situation. When the sheep were returned to field I on May 21, lamb no. 366 went along. Fourteen days later she broke a leg, and taken indoors died at the end of a week showing at autopsy one small, immature *M. expansa*. As figure 1 shows, continuously from May 21 until July 8 at least, tapeworm eggs were being deposited in the pasture. It is conjecture whether the worm in no. 366—an animal geophagic due to a heavy experimental infection with Haemonchus—came from paddock A due to recently passed eggs there, or from field I, due to its new supply of *M. expansa* eggs, or from infestation which escaped the plowing. The latter may easily have been possible for not all of field I is cultivable. What is striking, however, is that three susceptible animals placed in field I in July, September and October 1930 respectively failed later to show evidence of infection. This record of negatives goes over into 1931, when of four animals introduced in February, only the 3-year old Dorset ewe no. 332 later exhibited *M. expansa*, having contracted the tapeworm in late March or April.

Besides the above is one exceptional case. On May 21, 1930 there were placed in field I twelve ewes, none considered to be less than 5 years of age, and two lambs, all purchased locally from one farmer. The lambs had *M. expansa* when bought, and were removed from the pasture before the end of June. None of the ewes, on frequent examination throughout 1930, exhibited *M. expansa*, although four of them at purchase showed active infections with the second Moniezia species. The exception, ewe no. 396, was negative for *M. expansa* up to and including November 11, as figure 1 indicates. Specimens collected from pasture animals in December spoiled before examination, but on the next two examinations, January 7 and 16, she was positive after being indoors since December 22 for lambing. Evidently sometime between October 5 and December 4 she contracted infection from the pasture and may, in turn, have passed *M. expansa* eggs in field I before being taken indoors. Except for that possibility, this season, even under its drought conditions, would be another proved instance of long persistence of the pasture infestation, from early July 1930 to about April 1931. Conceding that no. 396 was passing the tapeworm eggs before being removed from the pasture in December, still indicates survival of the pasture stage over about 3 winter months.

The extension of the drought into 1931 brings out a point of interest in connection with the infections, which as figure 1 indicates were contracted in April. At least four animals are shown to have secured apparently light *M. expansa* infections definitely before a new crop of *M. expansa* eggs was being deposited in the pasture. This is not illogical despite the exceptional dryness of the preceding summer, because of an occurrence related to making fields I and II intercommunicating. On March 1, 1931 a night enclosure of wood panel fencing in the northeast corner of field I was removed, the animals being given a new night paddock at the corresponding corner of field II (marked N on map, Stoll, 1935). Despite occasional removal of accumulated manure from the field I night enclosure, there was in the nature of the case a concentration of any fecal borne infection in the soil of such a place, and to a lesser extent under the lowermost boards of the fence panels where it had been well protected. The grass growth here in the spring of 1931 would make otherwise hidden infestation available to grazing animals.

To be considered also is the possibility that such infections as were contracted before a new pasture infestation was established in May and June, 1931, may have come from the swampy area in field I. In a period of drought this would be utilized more for grazing, despite its non-succulent grasses.

A further point of interest concerning some of the animals introduced to field I in 1931 should be mentioned. Of the twelve which were placed in pasture February-May, six beginning in late June received monthly treatments with copper sulphate, indicated by ℞ in figure 1. It is an open question, in view of the whole situation, whether these treatments were responsible for some of the animals not securing *M. expansa*. Copper sulphate for no. 451 in mid-November, and tetrachlorethylene for no. 455 in mid-October appear to have interrupted the tapeworm egg outputs in these two cases, however.

DISCUSSION

While : literature on sheep parasites contains suggestions of the ability of *M. expansa* to overwinter on pasture, the only exact determinations appear to be those by Smith and Ring (1927) at this laboratory, and by Seddon (1931) in Australia. The former we have emphasized in the present article. Seddon had four experimental pens fenced in a Moniezia infested field. Pen A, with a permanent pool, was shown infestive in May, after being out of use 11 months. In pen B on sloping ground with no swamp, two uninfected animals contracted *M. expansa* in January (mid-summer) after the pen had been empty 6 months. Seddon also obtained infection in a lamb indoors which was fed 3 months later herbage cut with a lawn mower from pen B for seven successive days. This pen was protected by ditching against surface water contamination from the infested field. Pen B evidently much resembled the special enclosure in our field II, where overwintering was demonstrated.

Taken with our data, it is established that *M. expansa* infestation is well able to overwinter in the field. The fact that it successfully withstands two successive winters and the intervening seasons requires that it be looked upon as a distinctly hardy form in its external phase. That this is true in the presence of a certain amount of moisture is evidently equally true, for the literature has consistently indicated an

association of moist and even swampy ground as the accompaniment of infection with anoplocephalid tapeworms (see, for instance, Douthitt, 1915, and Jones, 1926). Conversely, Mönnig (1929) has reported a poor Moniezia season under drought conditions. This is in harmony with our 1930 experience, and with laboratory tests on viability of the eggs under dry conditions, as Scott and Honess (1931) report and we have verified for *M. expansa* and Obitz (1934) has found for *M. benedeni*, *Cittotaenia ctenoides*, and *Anoplocephala magna*.

The question as to the limiting time infestation can be retained in a pasture without adding new infection is not yet answered. One instance in our records is suggestive, but not clear-cut. Paddock A, upon which *M. expansa* eggs were being passed in 1924, and perhaps in 1925, was ploughed and reseeded in June 1926. No sheep were again present until the summer of 1928, but no *M. expansa* were again surely demonstrated to be infecting sheep in paddock A until 1931, following known deposition of new infection in 1930 as described. While the ploughing may have been the cause of burying the infestation over most of the paddock, the high permanent fence would prevent good marginal tilling. In these margins, which might be especially favorable for survival of the parasite and where grazing would none the less occur, the infestation could be expected to last a long time if capable of doing so. There is no evidence however that it did. Experiments to test the limits of the viable period are desirable, especially in relation to known degree of drying of the soil.

It should be clear, however, that knowledge of the pasture stage of *M. expansa* now covers three significant points which may permit effective restraint on this tapeworm. These are the demonstration by Daubney (1932) that the infective stage is ingested on pasture, by Stoll (1935) that this infective stage does not "migrate" from a pasture and is restricted apparently by gravity from going uphill, and by Smith and Ring (1927), Seddon (1931 a) and the author that an infestation persists in an unoccupied pasture for periods not only over one winter but two. From the standpoint of eradication of the pasture stage we find ourselves on the basis familiar in the consideration of other fecal borne parasites, such as the gastro-intestinal nematodes, except that Moniezia seems exceptionally resistant to destruction in the field. Unless our experience here with Moniezia is atypical, and

the several years observation involved would appear to nullify that factor, control measures against the unarmed tapeworm can be as adequately planned as if the abstruse steps in the life cycle were already clarified. The epidemiologic attributes of the parasite as now understood take into account its essential bionomics in the external phase, even in the absence of the final demonstration of the life history gap. Curtice (1934) significantly observes, "Tapeworms also have been reduced or entirely prevented in our experiments by pasture rotations. . . . The embryos, whatever their hosts, do not seem able to go far."

From figure 1 it may be seen that in the years 1926–32 there is direct evidence of the tapeworm being ingested on pasture during each of the months except January (and possibly February) for which we have since had a positive determination. Seddon (1931) found infections "contracted during mid-summer (January), autumn (March and April) and early winter (May)." Other authors, primarily from abattoir data, have implied year around infection. Curtice (1890) for the U. S. A. notes that "The broad tape-worm . . . may be found throughout all months of the year in localities where it occurs." Cameron (1923) found "ripe proglottides [of *M. expansa* and *M. planissima*] . . . throughout the year" in Scottish sheep. In Wales, Morgan (1924) observed "that infection with Moniezia spp. may take place very early in the year, if not all the year around" and Jones (1926) that both *M. expansa* and *M. planissima* "occurred throughout the year." Jenkins (1924) also concluded from observations made in Wales that "the period during which lambs became infected with Moniezia spp. is not confined to the spring, but may extend at least until August."

Neither our direct evidence, nor the indirect evidence obtainable by slaughterhouse examinations, need be taken to mean that all the months are equivalent as regards contraction of infection by susceptible animals, a necessary qualification which the above authors also bring out. Factors such as snow-covering during some winter periods and supplementary feeding of hay from ricks (which for our animals obtains during 5 months, November to April) would tend to insulate sheep from close contact with the pasture "floor" and thus from infestation residing there. It is nevertheless striking that no period

of the calendar is definitely out of consideration from the standpoint of sheep coming in contact with the infective stage in pasture. This fact, entirely harmonious with the idea of direct infection from contaminated soil, seems equally disharmonious with the usual conception of an intermediate host in the life history.

What additional observations of the present paper have a bearing on the life cycle puzzle of *M. expansa*? Three especially are of interest.

1. The initial infections of the 1929 season on pasture are striking in relation to the question of direct and indirect infection. With the facts in mind, (*a*) that no month of the calendar is excluded as a month in which *M. expansa* can be contracted by sheep, (*b*) that May in 1926 and 1927 proved infective after pasture survival of the infestation for 17 and 8 months respectively, (*c*) that sheep were in field I from October 1928 hence, and that thus supposititious intermediate hosts were presumably given opportunity to be attracted to the pasture,—nevertheless four successive susceptible animals on pasture early in May each grazed several weeks *before coming in contact* with the infestation. A somewhat sparse surviving infestation of tapeworm eggs on the "floor" of the pasture, directly infective when encountered, would appear to fit such facts almost to the exclusion of the alternative hypothesis. We have already noted that the period was "normal" as to climatic factors.

2. The primary point of this paper, persistence of the infective stage of this parasite on pasture, may well have more than suggestive value in considering the life history question. It is, for instance, readily understandable that an effective intermediate host would permit overwintering of the infestation in the field. For a pasture to remain infestive over two successive winters and the intervening seasons, however, puts a distinct strain on the idea that this persistence is within the body of a small intermediate host such as an insect. Supposedly that sort of an intermediate host would itself have a maximum lifetime of a year, probably much less. To assume an intermediate host larger than such a small invertebrate is incongruous with the feeding habits of a ruminant. Oppositely, survival of tapeworm eggs on pasture for long periods, to infect directly upon ingestion, could readily fit the fact of known survival of the infestation in the field over a second winter, provided evidence can be independently

adduced that the Moniezia egg is capable of surviving characteristic untoward environmental conditions. It can.

3. It should further be noted in regard to the demonstration of infectivity of field I in 1929 that the failure of susceptible animals in field II in 1928 to obtain the infection (Stoll 1935), when field I was patently infestive, is also against the pasture survival of *M. expansa* being within an intermediate host, for it failed to pass a separating wire fence under inviting circumstances in May and June 1928.

Earlier in this article emphasis was placed on the point that we were presenting our whole sheep experience of the years through 1932 in relation to the pasture phase of *M. expansa*. This, to us, is a point of considerable significance, for an understanding of the bionomics of this tapeworm must take account of a whole experience, as opposed to selected portions of it. In such a view, obviously a puzzling point is the fact that several animals, especially in 1930 and 1931, appear to have escaped infection in field I as judged by consistently negative fecal examinations, although other susceptibles did become infected. To entertain the idea that such animals avoided contact entirely with the infestation when other sheep with them got it is to go against knowledge derived either from direct observation of sheep habits, or the indirect evidence which infection with various sheep parasites furnishes of their close contact with the pasture "floor."

A choice remains among three possibilities. Either such animals did not happen to be examined when in the positive phase of the infection (which probably accounts for ewe no. 324), or if examined when positive the diagnosis was missed, or they did not exhibit mature *M. expansa*, the host having become immune and lost infection in advance thereof. All three possibilities are undoubtedly involved, although the second, we believe, least often. It so happened that in the author's sheep studies he had the good fortune, insofar as working with Moniezia is concerned, to observe lamb no. 299 early in its infection. This was the first demonstrated Moniezia-positive animal of the 1927 season. Interest aroused at that time became the occasion for noting the presence or absence of tapeworm eggs in examinations of all animals subsequently. It is this attention to the possible presence of the infection at all times, together with the fact that our adaptation

of Lane's flotation method (Stoll 1930) can be shown effective for demonstrating even small numbers of Moniezia eggs, which has furnished records whose study in perspective throws the present light on the characteristics of this parasite.

Finally, one item has been of much interest to the author. To examine in detail the epidemiological analysis put forward is to wish that here and there additional test animals or additional fecal or autopsy examinations had been available. Lack in such directions is more than compensated, however, by the objectivity of the data. To read the literature on the Anoplocephalidae is to realize how partial almost every observer has been to one of the two major alternatives in connection with the solution of the life history when weighing observational or experimental evidence. The fact that all the data of this paper were gathered during a period in which the author held almost completely to the indirect hypothesis, too, enhances—at least for him—the emphasis which it now conveys.

SUMMARY

1. Instances are detailed of the survival in the 1926–32 period of *M. expansa* on an infested pasture under conditions when sheep were not present, or if present were not harboring the tapeworm and thus not seeding the area with eggs.

2. These instances include the well-defined Smith and Ring report of 17½ months, and a probable case of 22 months, each of the latter periods spanning two winters.

3. Climatic factors of two survival periods are detailed.

4. An instance is analyzed of poor infectivity of the pasture during a period of drought.

5. It is shown that no month of the year is excluded as an infective period.

6. The bearing of the data on the eradication and life history problem of Moniezia is discussed.

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STUDIES ON FRENCHING OF TOBACCO[1]

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Among the so-called physiological diseases of tobacco, frenching is one of the most puzzling so far encountered by plant physiologists and pathologists. In Princeton this disease proved to be severe in the winter of 1932 in tobacco grown in greenhouses. During the following summer it occurred extensively in plants in an experimental field. Since that time numerous greenhouse experiments have been carried out in an endeavor, firstly, to prevent the disease, and secondly, to determine factors favoring its development. The object of this paper is to present the results of the experiments and such conclusions as could be drawn from the experimental data.

LITERATURE

Frenching of tobacco is very widespread in occurrence, as its presence has been observed in most of the large tobacco-growing areas of the world. In the United States frenching has been reported in Connecticut (4), Florida,[2] Georgia,[2] Indiana,[3] Kentucky[2] (5), Louisiana (13), Maryland[2] (13), Massachusetts,[2] New York,[4] North Caro-

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lina[2] (13), Ohio (13, 18), South Carolina,[2, 5] Virginia[2] (13), West Virginia,[6] and Wisconsin.[2] It has been reported in each of the following foreign countries: Canada,[7] France[2] (17), Italy (22), Russia[2] (17), Dalmatia[2] (17), Rhodesia (9), Transvaal (1), West Africa,[2] and Java[2] (17).

The earliest reference to frenching found in the literature was in a letter by John Clayton (6) to the Royal Society, May 12, 1688. Symptoms of frenching have been described by a number of early workers, including Dodge (5), Killebrew (13), and Allard (2). Clinton (4), in 1914, demonstrated that frenching cannot be transmitted to healthy plants by inoculation, thereby showing that it is a noninfectious disease and not a severe form of mosaic. His findings were corroborated by Wolf and Moss (26) in 1919, and by Garner (7) in 1922. Johnson (11) observed that poor soil aeration and low soil fertility were contributing factors favoring the development of the disease.

The suggestion that frenching is a nitrogen-deficiency disease was first advanced by Valleau and Johnson (23) in 1926. They concluded that it is the result of an accumulation of carbohydrates brought about by the absence of sufficient available nitrogen. This physiological condition was conceived to give rise to the development of new leaf tissue deficient in chlorophyll. In a later paper they (24) reported that frenching could be readily controlled by the addition of available nitrogen. This conclusion was confirmed by Haas (8) in California and by Hopkins (10) in Southern Rhodesia.

Morgan (16) in 1929 questioned the hypothesis that frenching is a nitrogen-deficiency disease. He was able to show that it developed when various fertilizer mixtures were added to the soil and was not restricted to mixtures deficient in nitrogen. In a later publication Valleau and Johnson (25) modified their original conception that frenching is a nitrogen-deficiency disease by suggesting that it is not caused directly by a deficiency of nitrogen alone, but probably by a

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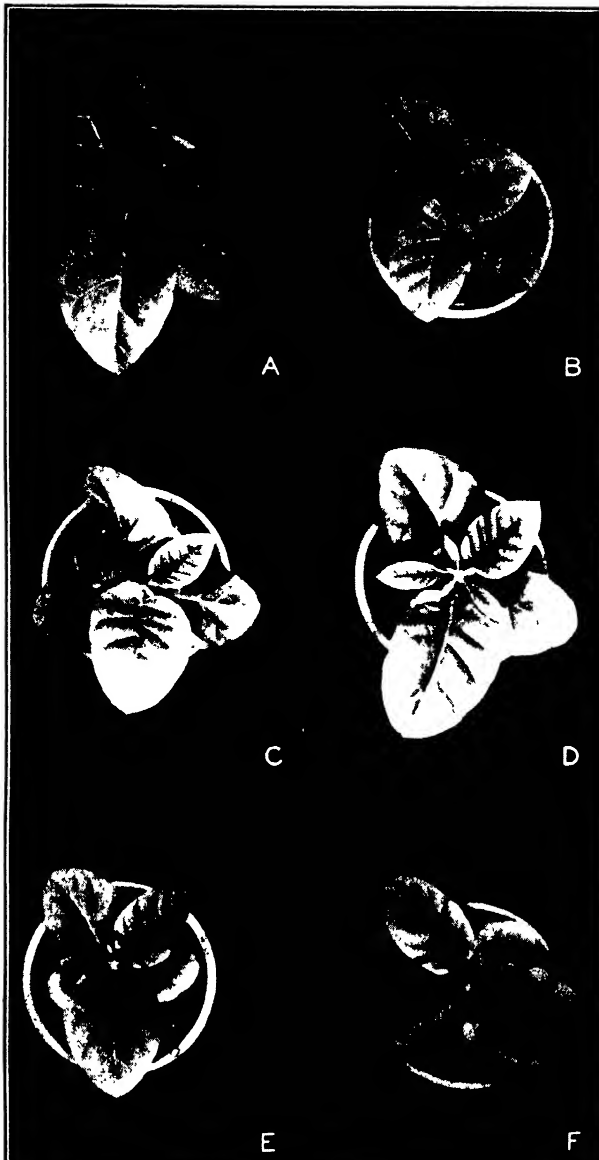
lack of balance between translocation of carbohydrates and of nitrogenous compounds within the plant. This condition may be brought about by a deficiency of any nutrient element essential for protein metabolism. In a more recent report Karraker and Bortner (12) suggested that, since frenching is obviously a disease of the growing points of the plant, its expression is the result of a retardation in the translocation of food materials to and from these growing regions.

The possibility that frenching is a toxicity disease rather than a deficiency disease was first expressed by McMurtrey (15) in 1932. He reported in a brief note that symptoms of thallium toxicity in solution culture resembled those characteristic of frenching, although not identical in all respects. Shear (19) also suggested that frenching might be a toxicity disease, but advanced no theory as to the nature of the toxic factor. No conclusive experimental evidence has been presented heretofore to show that frenching is a toxicity disease.

Symptoms

Since frenching has been adequately described by Clinton (4), Shear (19), and Valleau and Johnson (24, 25), only a brief description of the most characteristic symptoms will be given here. The earliest symptom to be observed is chlorosis along the margins of young leaves (Fig. 1, B). This gradually spreads toward the midribs and, finally, the entire interveinal regions are involved (Fig. 1, C). The network of veins, however, remains dark green. As the leaf develops, only the midrib elongates apparently, thereby producing a long narrow, ribbon-like or strap-shape structure (Fig. 1, D). Terminal growth is greatly retarded, resulting in a stunted plant with small, brittle, distorted leaves (Fig. 1, E). In severe cases the axillary buds are stimulated into growth, producing a rosette of small, narrow, strap-shape leaves (Fig. 1, F). These narrow leaves are very light in color. Frenching is not confined to the seedling stage of growth, but may appear at any stage up to flowering. It is sometimes accompanied by a yellowing of the lower leaves.

Symptoms of the disease are noticeable also in the roots. The diameter of the old roots is reduced to about one-half that of normal roots of the same age. The number of root hairs is markedly decreased; in some cases they are completely lacking. The cortex near



Photographed by J. A. Carlile

FIG. 1. Tobacco plants showing characteristic symptoms of frenching. A, healthy check plant; B, first symptoms with interveinal chlorosis along margin of leaf; C, interveinal chlorosis extending over entire leaf; D, elongating leaves, forming the strap-leaf stage; E, severe strap-leaf condition; F, stimulation of axillary buds forming a rosette of many, small, strap-shape leaves.

the root tips is pronouncedly discolored and necrotic. Root systems of frenched plants usually are more profusely branched than those of healthy plants.

EXPERIMENTAL

Numerous greenhouse experiments were carried out in an endeavor not only to control frenching of tobacco but also to determine its causal factor. Since frenching developed very severely in a near-by field, soil from this area was used in all of the experiments except where mention is made of soil from another source. This soil, which will be referred to as field soil, was quite heavy in texture, of high water-holding capacity, and of low fertility, with a neutral or slightly alkaline reaction.

An experiment was first carried out to determine what plants were susceptible to frenching. All seedlings were germinated in sand and then potted in field soil in 4-inch pots. In the first test, seedlings of 16 varieties of *Nicotiana tabacum* L. were used. These varieties, all of which developed frenching within 6 weeks, were Adcock, *angustifolia*, *auriculata*, Burley, *calycina*, *chinensis*, *colossea*, Connecticut Seed Leaf, *gigantea*, *latissima*, Little Orinoco, *macrophylla*, Maryland, *purpurea*, Turkish, and Zimmers. In the second series 18 different species of the genus Nicotiana were tested. The species that developed frenching were *N. alata* Link and Otto, *N. langsdorffii* Schrank, *N. longiflora* Cav., *N. rustica* L., *N. sanderae* Sander, and *N. sylvestris* Spegaz. and Comes. The following species showed no symptoms of frenching: *N. acuminata* Hook., *N. bigelovii* S. Wats., *N. caudigera* Phil., *N. clevelandii* A. Gray, *N. glauca* R. Grah., *N. glutinosa* L., *N. palmeri* A. Gray, *N. paniculata* L., *N. suaveolens* Lehm., *N. tomentosa* Ruiz and Pav., *N. trigonophylla* Dun., and *N. wigandioides* C. Koch and Fint.

Petunia hybrida Vilm. var. Henderson's Giant Empress, *Datura stramonium* L. (Jimson weed), and *Lycopersicon esculentum* Mill. var. Bonny Best (tomato) were the only other plants in the Solanaceae that showed characteristic symptoms of frenching. The chlorosis in these three species, however, was mild. The solanaceous plants that gave negative results were *Capsicum frutescens* L. (pepper), *Hyoscyamus niger* L. (henbane), *Lycium ferocissimum* Miers., *Nicandra physalodes*

(L.) Pers., *Physalis angulata* L. (ground cherry), *Solanum melongena* L. (eggplant), and *Solanum tuberosum* L. (potato).

None of the non-solanaceous plants grown showed any symptom of frenching. The species tested were *Zea mays* L. var. *rugosa* (field corn), *Fagopyrum esculentum* Gaertn. (buckwheat), *Beta vulgaris* L. (beet), *Spinacia oleracea* L. (spinach), *Vicia sativa* L. (vetch), *Glycine max* Merr. (soybean), *Medicago sativa* L. (alfalfa), *Phaseolus vulgaris* L. var. Early Golden Cluster (bean), *Tropaeolum majus* L. (nasturtium), *Gossypium hirsutum* L. (cotton), *Begonia semperflorens* Link and Otto (begonia), *Petroselinum hortense* Hoffm. var. *crispum* (parsley), *Salvia splendens* Ker. (scarlet sage), *Cucumis sativus* L. (cucumber), *Callistephus chinensis* Nees (aster), and *Lactuca sativa* L. var. *crispa* (lettuce).

A number of experiments were made in an endeavor to determine whether frenching is a parasitic, deficiency, or toxicity disease, since it seemed probable that it would fall into one of these three categories. Seedlings of *Nicotiana tabacum* var. Turkish were used as test plants in the experiments described below.

Parasitic Studies

Although Clinton (4), Wolf and Moss (26), and Garner (7) reported that frenching is not a parasitic disease, these investigators did not present the experimental evidence upon which their conclusions were based. Several experiments were made in an attempt to verify this conclusion. A microbiological examination of roots of field-grown healthy and diseased tobacco plants failed to show any association whatsoever between the disease and a pathogen. The following greenhouse experiments substantiated this observation.

When frenched plants were repotted in a mixture of 99 per cent sand and 1 per cent soil, they recovered temporarily within a few days, but became frenched again within 4 weeks. However, similarly diseased plants repotted in 100 per cent sand recovered and remained healthy till the experiment was discontinued. If the disease is due to a parasite, it is difficult to understand why those plants repotted in 100 per cent sand recovered permanently, whereas those placed in 99 per cent sand recovered only temporarily. Sufficient organisms should have been transferred on the roots of the frenched plants in the 100 per cent sand to cause a reoccurrence of the disease, if it is due to a parasite.

In another experiment roots from frenched plants were cut into small pieces and mixed thoroughly with washed sand. Healthy seedlings potted in this mixture and watered with tap water showed no evidence of the disease. When frenched plants were repotted in this mixture, they recovered and remained healthy. When a frenched plant was repotted in a pot of washed sand, in which a healthy plant was already growing, the healthy plant not only remained healthy but the repotted, diseased plant recovered and thereafter showed no symptoms of the disease.

Moreover, soil sterilization did not prevent the development of frenching completely, as would be expected if the disease were caused by a parasite. Soil was sterilized with steam at 15 pounds' pressure in an autoclave for 1 hour on 3 consecutive days. After the 3rd period of sterilizing, samples of soil were plated on agar plates. No growth of any sort developed on the plates within 14 days. Plants potted in this sterilized soil showed the first symptoms of frenching from 2 to 4 weeks later than they did in nonsterilized soil, but eventually all the plants became frenched. This delay in the appearance of symptoms may be explained as follows. Plants in sterilized soil grew faster than similar plants grown in nonsterilized soil, indicating that during the process of sterilization certain organic nitrogenous compounds might have broken down and thereby liberated nitrogen, which became available for plant growth. Therefore, the appearance of frenching was delayed until some of this nitrogen had been used up. The addition of small amounts of nitrogen to soil has been shown to retard the development of frenching. From this experiment and the 3 preceding ones, it appears improbable that frenching is a parasitic disease.

Physiological Studies

Studies were undertaken to determine whether frenching is a deficiency disease or a toxicity disease. Before these studies were carried out, however, it seemed advisable to ascertain the importance of such soil factors as texture, aeration, and reaction in making conditions favorable for the development of frenching.

In the first experiment dealing with soil texture, various amounts of peat and sand were mixed with field soil. Young seedlings were then potted in the mixtures in 4-inch porous clay pots and kept well

watered throughout the experiment. It was found that the incidence of frenching in various mixtures of sand and soil was equal to, if not slightly higher than, the incidence of disease in field soil alone. Peat and soil mixtures, on the other hand, lowered the incidence of frenching very noticeably. The number of plants that became frenched decreased as the proportion of peat in the mixture increased. When 50 per cent by volume of the mixture was peat, all plants remained healthy. Neither incidence nor severity of frenching could be correlated definitely with rapidity of growth. With another set of plants an experiment was made to determine the effect of variations in the amount of watering. When the plants were watered heavily, 75 per cent of them became frenched. With light watering, however, plants of a similar age remained healthy.

Since increasing proportions of peat in soil mixtures decreased and heavy watering increased the incidence of frenching, it was of interest to determine if aeration was a factor in the developing of frenching. One set of plants was potted in 100 per cent sand, and a similar set in 99 per cent sand and 1 per cent field soil by volume, in 4-inch pots placed in saucers. The saucers were kept partly filled with water at all times. Within 3 weeks after potting, all the plants in 99 per cent sand and 1 per cent soil became frenched, whereas those potted in sand alone remained healthy even when held under observation in a greenhouse for nearly 4 months. For all practical purposes the aeration and texture of the rooting medium in these two treatments were equal, indicating that frenching, under certain conditions, can develop even with good aeration.

The effect of soil reaction in promoting or retarding the development of frenching is brought out in the following experiment. Various amounts of pulverized lime were thoroughly mixed in a soil that does not produce frenching under ordinary conditions. Young seedlings were potted in these soil mixtures in 4-inch pots placed in saucers that were kept filled with water. The number of plants that developed frenching was observed at weekly intervals. None of the plants became diseased until the end of the 6th week. When the experiment was terminated after 11 weeks, the soil reaction was determined and the green weight of all plants measured. The experimental data in table 1 show that there was a definite correlation between the

hydrogen-ion concentration of the soil and the development of frenching. At pH 7.7 and 7.8 none of the plants became frenched, but at pH 7.9 and 8.0 nearly all the plants developed the disease. Above pH 8.0 a few plants became frenched, but the number decreased as the soil reaction became more alkaline. Even at pH 8.2 the plants made good growth, indicating that the absence of the diseased condition at this reaction was not due to poor growth. The range of soil reaction most favorable for the development of frenching was found to vary somewhat, depending upon other environmental conditions.

The preceding experiments show that soil texture, aeration, and reaction are important secondary factors in the development of frenching. They exert a pronounced action in modifying the incidence and severity of the disease.

TABLE 1

Effect of the Addition of Lime on the Development of Frenching

Amount of lime added gm./pot	Number of plants	Number of plants frenched after:						Records after 11 weeks	
		6 weeks	7 weeks	8 weeks	9 weeks	10 weeks	11 weeks	Soil reaction pH	Green weight of plants
0.0	5	0	0	0	0	0	0	7.67	5.0
0.5	5	0	0	0	0	0	0	7.80	7.1
1.0	5	1	1	1	3	5	5	7.90	10.5
1.5	5	2	2	4	4	4	4	8.00	10.0
2.0	5	1	2	2	3	3	3	8.13	14.1
3.0	5	0	0	0	1	1	1	8.23	12.3

Deficiency Studies. In order to determine if frenching might be a deficiency disease, a study was made of the effect on tobacco in sand cultures of a deficiency of the elements known to be essential to plant growth. The nutrient solution was added continuously by means of the constant-drip method of solution renewal (20). Typical symptoms of nitrogen, phosphorus, potassium, calcium, magnesium, and sulphur deficiencies were obtained, but none of these symptoms resembled those characteristic of frenching.

In order to determine the effect of the deficiency of some of these elements when seedlings were grown in soil, a repetition of the above experiment was made, using field soil instead of sand as the growing

medium. The nutrient solution was added twice each week, 100 cc. per pot. The plants became frenched when nitrogen or potassium was omitted from the nutrient solution added. In these particular deficiency treatments the plants grew very slowly. Typical symptoms of nitrogen- or potassium-deficiency, in addition to the characteristic symptoms of frenching, were obtained.

These experiments with sand and soil indicate that frenching does not result from a deficiency of any one of the principal essential elements. Since it occurred in soil with the addition of potassium in a nitrogen-deficient nutrient, and also with the addition of nitrogen in a potassium-deficient nutrient, neither a nitrogen nor a potassium deficiency can be considered as the factor causing the disease.

Experiments were then carried out to determine the effect of the elements known to be required for growth in small quantities, such as iron, boron, and manganese. Plants were grown in liquid nutrient cultures in which one of these elements at a time was omitted. None of the symptoms obtained, however, resembled those of frenching. Symptoms other than those of frenching were obtained when an excess of any one of these 3 elements was added. Various amounts of iron, boron, and manganese when added to soil failed to prevent the development of frenching or to bring about recovery when added to soil in which the plants were already frenched.

Since it has been shown by some workers (3, 14, 21) that copper exerts a stimulatory action on plants under certain conditions, tests were made on the effect of copper sulphate on the development of frenching. Table 2 gives the results of an experiment in which various amounts of $CuSO_4$ were added to field soil. The first treatment was made 5 days after the plants were potted. In group C the $CuSO_4$ exerted some toxic action as the plants were much smaller than those in group B. However, group A and B plants were about equal in size. In group A the symptoms of frenching were very severe. The plants in group B, however, showed only slight chlorosis after 20 days, but by the end of the experiment they appeared to be healthy again. Table 3 shows the effect of adding $CuSO_4$ to plants after they had become frenched. The plants in groups E and F recovered completely, while those in group D showed the rosette condition severely throughout the experiment. The experiments show quite clearly that

$CuSO_4$ had an appreciable effect in overcoming frenching and bringing about recovery from the diseased condition. The $CuSO_4$ exerted no measurable change on the hydrogen-ion concentration of the soil.

In order to determine if aluminum had any effect on the development of frenching, various amounts of aluminum sulphate were added to 4-inch pots of field soil. The results obtained when $Al_2(SO_4)_3$ was added at weekly intervals to young seedlings grown in soil are recorded in table 4. These data indicate that the doses of $Al_2(SO_4)_3$ added greatly retarded the development of frenching. Figure 2 shows

TABLE 2

Effect of Repeated Additions of Copper Sulphate on the Prevention of Frenching

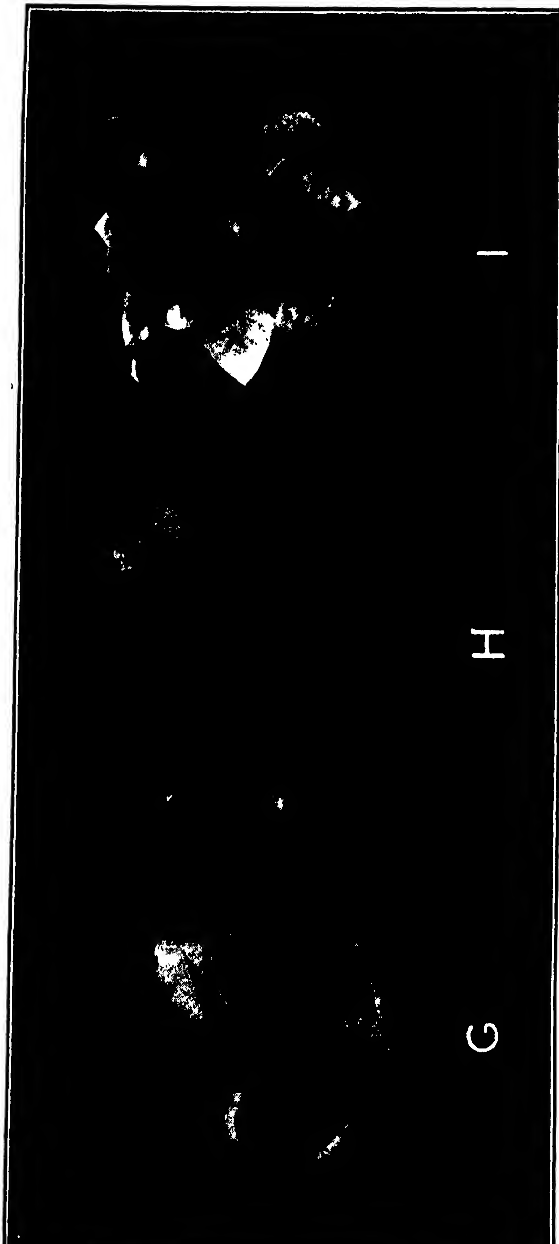
Treatment	$CuSO_4$ (1%) added each week (cc./pot)	Number of plants	Number of plants frenched after						
			16 days	17 days	20 days	35 days	50 days	65 days	80 days
A	0	5	3	5	5	5	5	5	5
B	5	5	0	3	5	5	3	1	0
C	10	5	0	1	2	2	1	0	0

TABLE 3

Effect of Repeated Additions of Copper Sulphate on the Recovery of Plants from Frenching

Treatment	$CuSO_4$ (1%) added each week (cc./pot)	Number of plants	Number of plants frenched after				
			16 days	35 days	50 days	65 days	80 days
D	0	5	5	5	5	5	5
E	5	5	5	5	4	2	0
F	10	5	5	3	2	0	0

a plant from each of groups G, H, and I, as it appeared 42 days after potting. The extent to which $Al_2(SO_4)_3$ retarded frenching varied with the number of weekly treatments. Thus, with only one treatment, the first appearance of the disease was delayed nearly 2 weeks. With 2 treatments at weekly intervals, disease symptoms did not appear till 4 weeks after those in the check pots. With 3 treatments, only 1 plant out of 5 developed frenching within 10 weeks. The data in table 4 also show that the addition of $Al_2(SO_4)_3$ lowered slightly the reaction of the soil. $Al_2(SO_4)_3$ had only a slight depressing influence



Photographed by J. A. Carlile

FIG. 2. Plants showing the effect of aluminum sulphate on prevention of frenching. G, check plant on left showing severe frenching; H, plant that received 1 gram $Al_2(SO_4)_3$ showing mild chlorosis; I, plant that received 2 grams $Al_2(SO_4)_3$ showing no frenching. Faint chlorosis appeared on plant I 3 weeks after plants were photographed.

on growth. The apparent increase in growth with small amounts of $Al_2(SO_4)_3$, as shown in table 4, was due to the stunting that accompanies the development of frenching.

The addition of $Al_2(SO_4)_3$ was beneficial also in bringing about recovery from the frenched condition. In this experiment, the data from which are given in table 5, all of the plants were frenched before the first addition of $Al_2(SO_4)_3$ was made. Recovery was partial when only 1 or 2 grams of $Al_2(SO_4)_3$ were added per pot, but was complete when 3 grams were added in 1-gram quantities at weekly intervals. The degree to which $Al_2(SO_4)_3$ promoted recovery is shown by the

TABLE 4

Effect of Aluminum Sulphate on the Development of Frenching of Tobacco in Field Soil

Group	$Al_2(SO_4)_3$ (1 gm./pot) added at weekly intervals for: (weeks)	Number of plants	Number of plants frenched after: 30 days	35 days	44 days	54 days	60 days	70 days	Records after 70 days: Soil reaction (pH)	Green weight of plants (gm.)	Remarks
G	0	5	1	5	5	5	5	5	6.80	8.4	Severe rosette
H	1	5			2	5	5	5	6.57	9.3	Chlorosis
I	2	5					1	2	6.45	11.6	Slight chlorosis on 2 plants, rest healthy
J	3	5						1	6.40	7.8	Healthy
K	4	5						0	6.25	7.1	Healthy
L	5	5						0	6.15	5.8	Healthy

plants in figure 3, photographed 48 days after first treatment. Thus, aluminum was effective not only in preventing the development of frenching, but also in bringing about recovery after the plants had become diseased. Similar results were obtained when $AlCl_3$ was substituted for $Al_2(SO_4)_3$.

To test the effect of aluminum and copper on the plant itself, young tobacco seedlings were potted in soil in 2-inch pots placed in glass saucers that were kept filled with water. After some 3 weeks all the plants became frenched; the roots by this time were extending down through the hole in the bottom of the pot. The pots were then suspended in glass tumblers. When the extending roots were submerged

in a dilute nitrogen solution, the plants recovered. However, when the roots were submerged in a dilute solution of $Al_2(SO_4)_3$ or $CuSO_4$, recovery was only partial.

Inasmuch as frenching failed to develop on plants grown in sand and water cultures without the addition of both copper and aluminum, it is probable that the cause of frenching cannot be considered as a deficiency of either of these two elements, even though they had a pronounced action on the disease. The beneficial effect obtained by adding $Al_2(SO_4)_3$ or $CuSO_4$ to the soil probably was due to some change in the soil complex, rather than to any direct effect of the aluminum or copper on the plant itself.

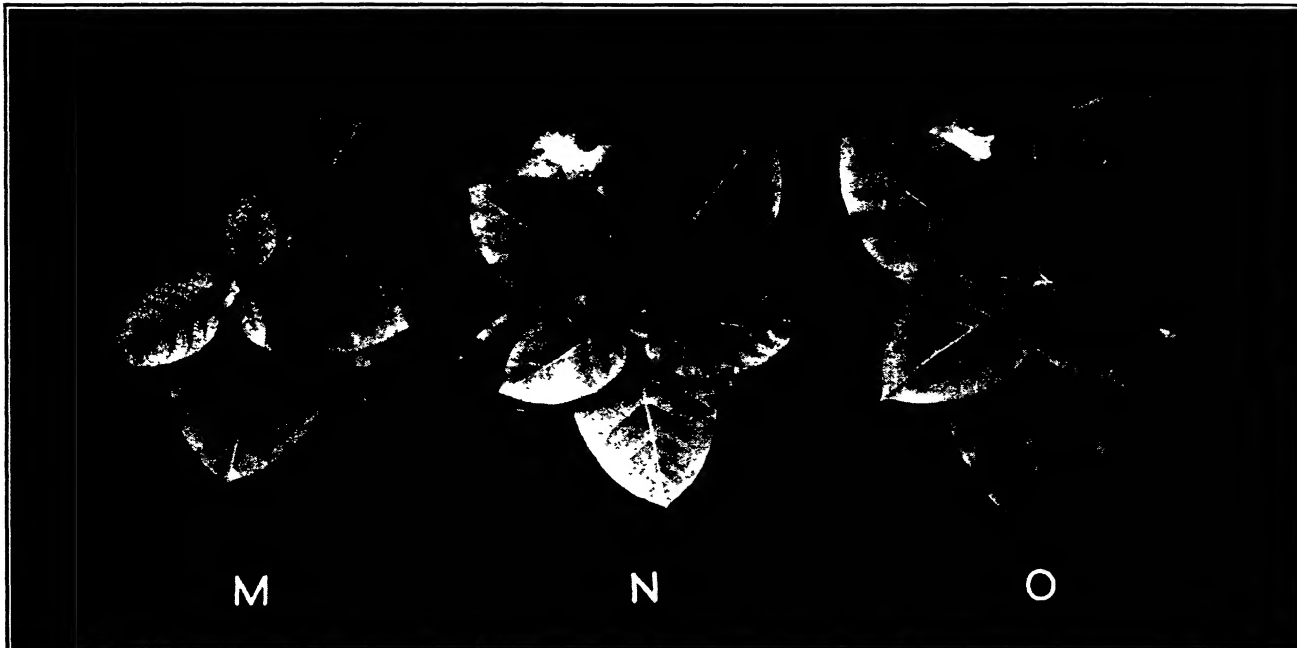
TABLE 5

Recovery from Frenching by the Addition of Aluminum Sulphate to the Soil

Group	$Al_2(SO_4)_3$ (1 gm./pot) added at weekly intervals for: (weeks)	Number of plants	Number of plants frenched after: 13 days	18 days	23 days	28 days	33 days	48 days	Records after 48 days: Green weight (gm.)	Soil reaction (pH)	Remarks
M	0	10	10	10	10	10	10	10	4.8	7.10	Severe rosette
N	1	10	10	10	10	10	10	10	6.1	7.05	Chlorosis
O	2	10	9	9	7	5	5	4	6.1	6.85	Faint chlorosis on 4 plants, rest healthy
P	3	10	9	6	4	2	0	0	5.9	6.70	Healthy

These experiments on mineral deficiencies indicate definitely that frenching is not caused by a deficiency of nitrogen, phosphorus, potassium, calcium, magnesium, sulphur, iron, boron, or manganese. Since these elements are the only mineral elements commonly considered as essential to plant growth, it is probable that frenching is not directly caused by a deficiency of any mineral nutrient.

Toxicity Studies. Since it was impossible to demonstrate that a deficiency of any of the essential elements is the cause of frenching, the next step was to determine if frenching is a toxicity disease. Experiments were carried out in which such factors as soil complex and mineral nutrition were eliminated as far as possible. In the first experiment seedlings were grown in washed quartz sand in 4-inch pots placed in saucers. One set of seedlings was watered with tap water and a simi-



Photographed by J. A. Carlile

FIG. 3. Plants showing effect of aluminum sulphate on recovery from frenching. All plants showed frenching before experiment was started. M, plant that received no $Al_2(SO_4)_3$ showing severe frenching; N, plant showing partial recovery after receiving 1 gram $Al_2(SO_4)_3$; O, plant showing complete recovery after receiving 2 grams $Al_2(SO_4)_3$ at weekly intervals.

lar set watered with a water extract of field soil. This extract was prepared as follows. Approximately 7 kilograms of soil were placed in a 5-gallon carboy and mixed thoroughly with about 15 liters of distilled water. When the soil had settled to the bottom of the carboy, the liquid was siphoned off and used in watering the plants. The plants watered with soil extract became frenched in about 7 weeks, whereas those watered with tap water were still healthy when the experiment was discontinued 5 weeks later.

In another experiment plants were grown in sand with various proportions of field soil. The experimental data are given in table 6. These results show that it is possible to bring about the development

TABLE 6

The Incidence of Frenching in Sand Mixtures Containing Very Small Amounts of Field Soil

Proportion of field soil added to sand		Number of plants	Number of plants frenched after							
Per cent	Parts per thousand		3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	9 weeks	23 weeks
0.0	0.0	5	0	0	0	0	0	0	0	0
0.05	0.5	5	0	0	2	3	3	4	5	
0.5	5.0	5	0	0	1	3	3	5		
0.75	7.5	5	0	0	2	5				
1.0	10.0	5	0	1	2	5				
10.0	100.0	5	4	5						

of frenching in plants grown in quartz sand by the addition of as little as 1 part of field soil to 2000 parts of sand. Plants grown in sand to which no soil was added remained healthy and showed no symptoms of frenching.

It was later found possible to produce mild symptoms of frenching in plants grown in washed quartz sand without the addition of any soil whatsoever. Plants were grown in quartz sand in 4-inch porous clay pots placed in saucers kept partly filled with tap water from a deep well. At the end of 5 months the plants, still healthy, were practically dormant, and were replaced by new seedlings. This second set of plants showed the chlorotic stage of frenching within 2 months after potting. These results may be explained as follows. The toxic

material probably was present in the tap water in extremely small concentrations. With repeated watering of the plants from the bottom of the pots and continual evaporation of water from the pot and sand, this toxic material gradually accumulated in the sand. By this time the first set of plants had become nearly dormant and showed no evidence of toxicity. However, when new plants were planted in this sand and new growth started, the toxicity was made evident by the typical chlorosis characteristic of frenching in the tip leaves.

The 3 experiments described above would indicate that frenching is a toxicity disease rather than a mineral deficiency disease. The toxic principle was apparently added in the first experiment with the soil extract and in the second experiment with the trace of soil. Further experiments to determine the nature of the toxic principle are under way.

DISCUSSION

Although the experimental evidence presented in this paper indicates that frenching is a toxicity disease, this cannot be considered as conclusively established until the actual cause of the disease has been demonstrated. It is possible that the deficiency of some element, not recognized as essential to growth but nevertheless required in minute amounts and present as an impurity, may be the cause of frenching.

The experimental data have given no indication as to how the plant is affected by the causal agent of frenching. Therefore, it is difficult to explain how nitrogen, aluminum, or copper acts in preventing the development of frenching. These minerals may act directly on a toxic principle or indirectly by affecting some condition necessary for the liberation of such a principle. Soil texture, soil aeration, soil reaction, and mineral plant nutrition were found to be important secondary factors capable of modifying the incidence of the disease.

Further work may lead to a satisfactory explanation of the high susceptibility of certain Nicotiana species and the low susceptibility of many other species grown under similar conditions. It is possible that the Nicotiana species affected may be highly sensitive to some toxic element, since it is well known that plants differ in their tolerance of poisons.

The experimental evidence does not permit the formulation of any

definite hypothesis as to the cause of frenching. However, the evidence does reveal some facts concerning the nature of the disease and the means by which it may be controlled.

SUMMARY

In the genus Nicotiana frenching developed severely in *N. alata*, *N. langsdorffii*, *N. longiflora*, *N. rustica*, *N. sanderae*, *N. sylvestris*, and in 16 varieties of *N. tabacum*, but not in 12 other species of Nicotiana grown under similar conditions. Of the 26 other solanaceous and non-solanaceous species tested, only *Datura stramonium*, *Lycopersicon esculentum*, and *Petunia hybrida* showed chlorosis characteristic of frenching.

Frenching in tobacco in a greenhouse was controlled by soil composting, the addition of peat, repeated applications of a nitrogenous fertilizer, and by several applications of a dilute solution of copper sulphate or aluminum sulphate.

No association was found between frenching and any pathogenic organism.

Experiments on the deficiency of each of the elements essential to plant growth failed to produce symptoms that resembled those of frenching.

Frenching was produced in plants grown in sand by the addition at daily intervals of a water extract of field soil. It also was produced by adding as little as 1 part of field soil to 2000 parts of sand. In young seedlings, in sand, it was produced by the addition of tap water from a deep well, over a long period of time.

The experimental evidence indicates that frenching probably is not a mineral-deficiency disease, but a toxicity disease produced by some toxic principle that is present in certain soils and that exerts its toxic action only under definite environmental conditions.

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